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IN THE U.S. PATENT AND TRADEMARK OFFICE

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| Inventor | Martin GEIER et al | |
| Patent App. | 10/517,675 | |
| Filed | 20 July 2005 | Conf. No. 1884 |
| For | COMPOSITION FOR ATTRACTING BLOOD SUCKING ARTHROPODS AND FR | |
| Art Unit | 1616 | Examiner Fisher, A |

Hon. Commissioner of Patents
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DECLARATION UNDER 37 CFR 1.132

I, Martin GEIER, a citizen of Germany, residing at Blaue Stern Gasse 7, 93047, Regensburg, Germany, declare as follows:

THAT I am the same Martin Geier who executed a Declaration Under 37 CFR 1.132 on 22 February 2009 and that Applicants made of record on 3 March 2009;

THAT I am aware that the Examiner has cited Bosch et al, "Contribution of Fatty Acids to Olfactory Host Finding of Female Aedes aegypti", Chem Senses 25: 323 to 330, 2000, for its disclosure that insect attracting compositions may contain lactic acid, valeric acid and ammonia, and that valeric acid and caproic acid are adjacent homologs;

THAT in order to demonstrate that the insect attracting compositions according to the present invention having lactic acid, caproic acid, and ammonia, in the respective molar ratios of 5:3:9 and 1:2:0.3 possess a surprisingly and significantly higher level of insect attractiveness than the corresponding compositions having lactic acid, valeric acid and ammonia, I have either personally conducted or supervised the carrying out of the following tests:

Test Protocol

For each test, about 20 female A. aegypti mosquitoes were used from cultures of the University of Regensburg, 7 - 23 days old, which had no blood meal before. Details of the olfactometer and the experimental procedure are described elsewhere (Geier, M. & Boeckh, J. *Entomol. Exp. appl.* 92, 9-19 (1999); Geier, M. Bosch, O.J. & Boeckh., J. Chem. Sens. 24, 647 - 653 (1999). At stimulus onset, the mosquitoes in the start chamber are set free to fly through the olfactometer. During their zig-zag flight through the straight tube they encounter alternately air streams from either arm.

Following the favored air stream during upwind flight the mosquitoes enter the respective upwind chamber where they are counted 30 s after stimulus onset. The number of mosquitoes attracted to the chamber serves as the measure for the attractiveness of the odor stimulus. Odor stimuli were produced by passing purified air through Erlenmeyer flasks which contain the odor solution (Geier, M. Bosch, O.J. & Boeckh, J. *Chem. Sens*, 24, 647 - 653 (1999); Geier, M. Bosch, O.J. & Boeckh, J. *J. Exp. Biol.* 202, 1639 - 1648 (1999). The relative concentration of each odor stimulus is given with respect to their molar mixing ratios in the gaseous phase.

Stimuli: La = Lactic acid, Am = ammonia, Va = Valeric acid, and Ca = Caproic acid. Values from 20 tests per treatment are lumped together. Treatments were tested in random order.

Table 1: Attractiveness of different blend compositions for yellow fever mosquitoes *Aedes aegypti* in a Y-tube bioassay.

| Stimulus | Number of tested mosquitoes | Number of mosquitoes in the test chamber | Attracted mosquitoes in % |
|---|---|---|---|
| La1:Ca2:Am0.3 | 401 | 359 | 89.5 |
| La5:Ca3:Am9 | 398 | 305 | 76.6 |
| La1:Va2:Am0.3 | 411 | 227 | 55.2 |
| La5:Va3:Am9 | 401 | 189 | 47.1 |
| La1 | 389 | 87 | 22.4 |
| Am0.3 | 407 | 9 | 2.2 |
| Ca2 | 403 | 14 | 3.5 |
| Va2 | 399 | 19 | 4.8 |
| La5 | 402 | 75 | 18.6 |
| Ca3 | 390 | 12 | 3.1 |
| Va3 | 388 | 17 | 4.4 |
| Am9 | 399 | 10 | 2.5 |

**Commentary:**

As can be seen from the data the replacement of Valeric acid with Caproic acid leads to a dramatic improvement in attractiveness.

This is more easily seen if the data is displayed differently:

Table 2: Comparison of attractiveness of different blend compositions for yellow fever mosquitoes *Aedes aegypti* in a Y-tube bioassay

| Stimulus | Attracted mosquitoes in % | Relative attractiveness in % |
|---|---|---|
| La1:Va2:Am0.3 | 55.2 | 100 |
| La1:Ca2:Am0.3 | 89.5 | 162 |
| | | |
| La5:Va3:Am9 | 47.1 | 100 |
| La5:Ca3:Am9 | 76.6 | 163 |

THAT based upon the above I conclude that the level of insect attractiveness achieved according to the combination of lactic acid, caproic acid and ammonia in the stated molar ratios of 5:3:9 and 1:2:0.3 is surprisingly and significantly higher than it is when the prior art compositions containing a combination of lactic acid, valeric acid and ammonia were applied as insect attractants in the same molar ratio.

THAT the increase in attractiveness achieved by substituting caproic acid for valeric acid to obtain the combination of lactic acid, caproic acid and ammonia in the stated molar ratio of 5:3:9 over the prior art composition amounted to a 63% increase in attractiveness, and by substituting caproic acid for valeric acid to obtain the combination of lactic acid, caproic acid and ammonia in the stated molar ratio of 1:2:0.3, over the

prior art composition amounted to a 62% increase in effectiveness, was surprisingly high and I did not expect to obtain such a high level of increase;

THAT I am aware of no data inconsistent with those presented above or which would lead one to a contrary conclusion; and

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 USC 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2/2/2010
Date

Martin Geier

*Entomologia Experimentalis et Applicata* 92: 9–19, 1999.



# A new Y-tube olfactometer for mosquitoes to measure the attractiveness of host odours

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*Key words:* mosquito, *Aedes aegypti*, host seeking behaviour, lactic acid, carbon dioxide, host odour, attractant, olfactometer

**Abstract**

In a new bioassay a small Y-shaped wind tunnel is used to quantitatively investigate the responses of mosquitoes to host odours. Female yellow fever mosquitoes, *Aedes aegypti* (L.) (Diptera: Culicidae) were tested to (1) a human hand, (2) an extract with human skin residues, (3) L-(+)-lactic acid, and (4) carbon dioxide. The responses to the skin extract followed a sigmoid dose response curve. The most effective dose attracted 80–90% of the mosquitoes within 30 s and was as effective as the human hand. L-(+)-lactic acid was identified in this extract and found attractive for mosquitoes also when presented alone. Carbon dioxide stimulated taking flight and was attractive, an effect which was synergistically enhanced in combination with L-(+)-lactic acid. The presented bioassay is especially suited to test the behavioural effects of synthetic odours as well as of natural odour sources. Due to the fast response of the mosquitoes, the sensitivity, and the simplicity of the testing procedure it is a potent tool in the search for new attractive components.

## Introduction

Olfaction plays an important role in host recognition and orientation of female mosquitoes (Sutcliffe, 1987; Takken, 1991; Davis & Bowen, 1994). Yet, only a few of the odours are known, which are used by mosquitoes for host selection and location. Carbon dioxide, a compound universally emitted with the breath by all kinds of potential hosts, stimulates mosquitoes in that it increases flight activity and duration of flights; it is also an attractant (Gillies, 1980; Eiras & Jepson, 1991). L-(+)-lactic acid, present on human skin as well as in human breath, was repeatedly shown to attract *Ae. aegypti*, but only in combination with carbon dioxide (Acree et al., 1968; Smith et al., 1970; Eiras & Jepson, 1991, 1994). Recently it has been demonstrated that a synthetic mixture of fatty acids identified in Limburger cheese and human sweat attracts *An. gambiae* in a wind tunnel (Knols et al., 1997). Some amines, estrogens, amino acids, and alcohols were also reported to attract mosquitoes, but many of these results remained contradictory. Moreover, mixtures of such compounds never matched the effect of the natural host odour (summaries in Hocking, 1971; Takken, 1991). To identify new attractants and to explore the composition of the attractive natural host odour we have designed a new bioassay, which was supposed to meet the following requirements: (1) Simple and fast testing of many odour samples in a limited time, (2) Easy comparison of extracts from natural odour sources or synthetic attractants with the authentic, natural host odour (Miller & Strickler, 1984), (3) Monitoring of all behavioural sequences in the host finding process as are perception, activation, orientation towards the odour source, and landing (Sutcliffe, 1987), (4) Wide measuring range to differentiate the strength of attractive stimuli, (5) Easy control and avoidance of contamination caused by previous stimuli (Schreck et al., 1967, 1981). In order to prove whether this bioassay fulfils these requirements, the following stimuli were tested: a human hand, an extract from human skin residues, L-(+)-lactic acid, and carbon dioxide. The extract's effectiveness was characterised by dose-response measurements. To as-

sure that no important kairomones were lost in the course of the sampling of active material, we sampled the skin residues from a single person and compared their attractiveness with the one of the same person's hand.

We have chosen the yellow fever mosquito *Aedes aegypti* (L.) for our experiments because this species has been already studied in many laboratories and a substantial amount of knowledge exists about this insect (Takken, 1991, 1996; Davis & Bowen, 1994).

## Materials and methods

### *Animals*

Female *Ae. aegypti* (10–40 days old), from cultures in the Centre for Plant Research (*Pflanzenschutzzentrum*) at Bayer AG in Monheim (Germany), were used in our experiments. The larvae were fed with Tetramin® fish food. The adult females and males were kept together in a container under the following conditions: 26 °C–28 °C; 60%–70% r.h.; L12:D12. They had constant access to a 10% glucose solution on filter paper.

### *Bioassay*

*Experimental set-up.* The Y-tube olfactometer consisted of a transparent plexiglass tube (inner diameter: 7 cm, wall thickness: 0.5 cm; Figures 1, 2). Removable chambers were located at all three ends (inner diameter: 7 cm, length: 10 cm, thickness: 0.5 cm). One end of a chamber was covered with gauze while the other had a rotating gauze screen (Figures 1, 2). Both chambers on the branches of the Y-tube fit into PVC® tubes (inner diameter: 10 cm, length: 20 cm) where the odour stimuli are presented (Figures 1p, 2). Air from the institute's pressurised air system was cleaned with a charcoal filter (Figure 1b) before introducing it into the testing apparatus. A bath filled with deionized water was used to humidify the air (Figure 1e). The water was kept at a constant temperature of 60 ± 2 °C by a heating coil with thermostat (Figure 1d). The ratio of moist and dry air was regulated (Figure 1h) so that a certain humidity could be maintained. Before introduction into the stimulus tubes, the moist air was warmed with heating elements (Figure 1l). Temperature in the olfactometer was 28 ± 1 °C, the relative humidity was 70 ± 5%. These values were constantly monitored by a thermohygrometer (Conrad Electronics, Wernberg, Germany) and regulated manually. The temperature of the testing room was 22–26 °C. Pressurised air was permanently introduced into the olfactometer at a rate of 80 l/min. The wind speed in the tunnel, measured using a thermistor, was 0.2–0.3 m/s in the branches and 0.4–0.6 m/s in the stem of the Y-tube. A ventilator (Figure 2m) in front of the stimulus tubes was switched on after the experiment to reverse the air flow in the olfactometer (flow rate in Y-stem: 0.4–0.6 m/s) so that the mosquitoes could be lured back into the release chamber using the hand as an attractant. The olfactometer was placed on a white table, a white cardboard shield of 150 cm × 50 cm was placed on each side to prevent optical stimulation by the experimenter. Two fluorescent light tubes (40 W) served for overhead illumination. No additional visual stimuli were presented.

*Behavioural test.* For a test 20 female mosquitoes were lured out of their container into the release chamber using the human hand as a bait. This procedure ensured that all mosquitoes used in the test were ready to seek for a host. Five minutes after the release chamber had been attached to the olfactometer, the test stimulus was presented in one arm and the control stimulus in the other. At the same moment the release chamber was opened and the mosquitoes were allowed to enter the tube. Thirty seconds later, the rotating screens were closed and the numbers of mosquitoes counted, which were trapped in the release-, test-, and control chamber, respectively. Then the ventilator (Figure 2m) was switched on to lure the mosquitoes back into the release chamber. In this way we determined whether the mosquitoes were still ready to respond to the natural host. Generally a test took no more than 6 min. The time period between two tests for one mosquito group was at least 30 min. Control tests after previous odour stimulation were repeatedly used to check for contamination (see results). In preliminary experiments we had observed strong contamination effects when the inner walls of the olfactometer were touched with the hands; therefore this was carefully avoided in further experiments. As soon as contamination was detected, the olfactometer was thoroughly cleaned with detergents, water, and ethanol. Tests ran from 9:00 a.m. to 6:00 p.m.

Six experiments were conducted independently from each other with different groups of mosquitoes. Within an experiment the groups were randomly tested to each treatment, whereby a given mosquito group was exposed to all treatments. In successive tests the test stimulus was alternately offered in each arm of the olfactometer.

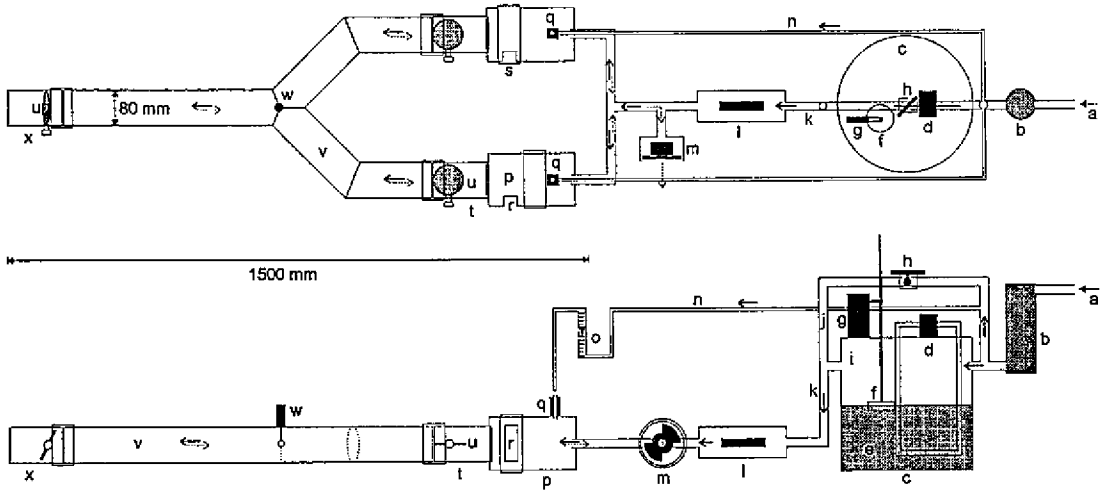


*Figure 1.* True to scale construction plan of the experimental set-up. Top view (upper picture) and side view (lower picture): pressurised air (a), active charcoal filter (b), stainless steel water container 300 × 300 × 1.5 mm (c), thermostat with heating coil (d), deionized water (e), floater (f) with switch (g) for regulating the water level, valve (h) for adjusting the ratio of moist (i) and dry air (j), plastic tube 23 mm × 25 mm for air supply (k), heating element (l), ventilator (m) for reversing the air stream after completing an experiment, tube for stimulus air stream (n), flow meter (o), stimulus tubes (p), temperature regulated heater with glass cartridge (q) for stimulus application, opening for hand (r), closure for hand opening (s), test and control chamber (t), rotating nylon screen (u), perspex Y-tube (v), temperature and humidity indicator (w), release chamber (x) with rotating screen (u). The arrow indicate standard direction of the air stream during an experiment. In this situation the ventilator (m) is off. The dashed lines symbolise the air stream which has been reversed after the ventilator (m) has been turned on.

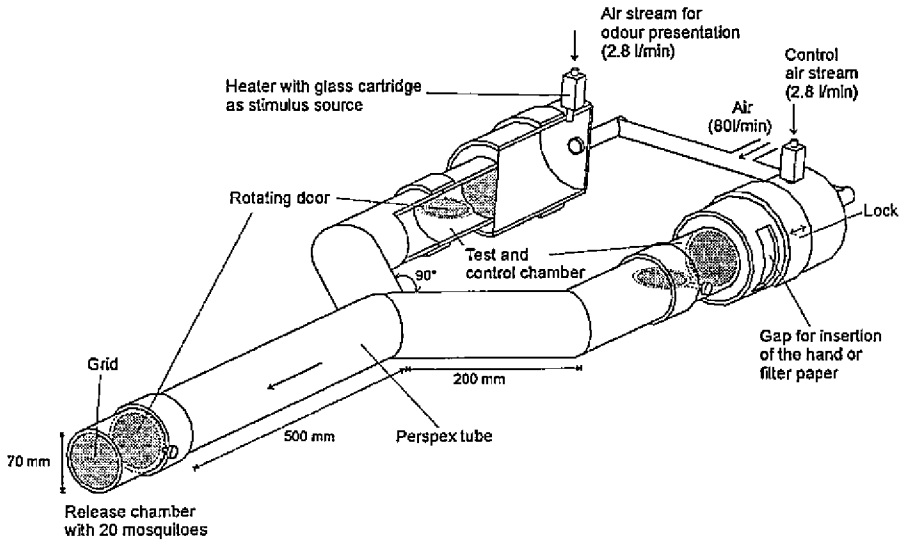


*Figure 2.* Lay-out of the Y-tube olfactometer.

*Odours, stimulus delivery, and test program*

*Natural host.* The four fingers of the experimenter's hand were introduced into the olfactometer through a gap in the stimulus tube (Figure 2). This led to an increase of the air temperature in the test arm by 0.2 ± 0.2 °C. Two hours before, the experimenter's hands were rinsed with water, no cosmetics or perfumes were used. The hand of the same person was used in all experiments. In experiment 1 the hand was tested once in a month over altogether 3 months to monitor possible variations of the attractiveness of this standard stimulus over a longer period (see Table 1 for arrangement of stimuli).

*Fluid samples.* Liquid test substances were presented either on filter paper or in glass cartridges: (1) 1 ml of a test solution was pipetted onto a filter paper of 7 cm diameter. After the evaporation of the solvent, the paper was fixed in the middle of the stimulus tube by means of a metal clamp. To avoid contamination, a new clamp was used in each test. A clean filter paper with 1 ml solvent served as control. (2) The test solution was pipetted to the inner side of a glass cartridge of 3.3 mm inner diameter and 5 cm length. After the solvent had evaporated, the cartridge was placed into the heating element on the stimulus tube (Figures 1, 2q). The function of the heater was to increase the evaporation of the test substances. The odour was delivered by blowing air through the cartridge at a rate of 2.8 l/min. This caused an increase of air temperature in the test arm by 0.2 ± 0.1 °C. A cartridge with solvent (airflow: 2.8 l/min; heater: 60 °C) served as control. The flow rate of air streams were controlled by flow meters (Rota, Figure 1o).

*Skin extract.* Odour samples from the person, whose hand had been tested, were obtained by rubbing the hands, forearms, feet, and calves with cotton pads soaked in ethanol (p.A. Fluka, Germany). Each body area was rubbed 20–30 times for 5 min with a separate pad. The pads were then extracted in glass columns of 5 mm inner diameter and 100 mm length with methanol (p.A. Fluka, Germany) at a flow rate of 0.5 ml/min. Preliminary tests revealed that most of the effective material was extracted within the first 5 ml of solvent. Extracts from 50 pads obtained during a period of 2 months were combined, concentrated to 30 ml by evaporation in a rotary evaporator, and then centrifuged at −20 °C with 3000 rpm for 2 h, yielding a clear yellow supernatant. A blank extract made from 50 clean cotton pads served as control. In experiment 2 (using filter papers for stimulation) and in experiment 3 (using glass cartridges for stimulation) different doses of skin extract were tested (see Table 1). In experiment 4 the skin extract was tested against a hand using glass cartridges for stimulation, because the hand as well as the heated cartridges deliver a comparable thermal stimulus (see Table 1).

*Lactic acid.* The test solution consisted of 8 mg L-(+)-lactic acid (99%, Fluka, Germany) dissolved in 100 ml methanol (p.A., Fluka, Germany). This solution was tested in experiments 5 and 6 (see Table 1).

*Gaseous samples. Carbon dioxide.* The pure gas (99.9%, Linde, Germany; flow rate: 50 ml/min) from a cylinder was mixed with clean air and passed through the heating element while the total airflow through the heater was kept constant at 2800 ml/min. The flow rate of the gas was measured by a flow meter (Rota). The resulting concentration of carbon dioxide in the test chamber was measured with a carbon dioxide analyzer (LI-6251, Li-Cor, Lincoln; Nebraska, U.S.A.) and found at 0.1%. The air in the control chamber contained 0.035% carbon dioxide, the amount generally found in the atmosphere. To prove the synergistic effect between lactic acid and carbon dioxide both stimuli were tested in experiment 6 (see Table 1).

To estimate how the odours are distributed in the olfactometer we visualised the stimulus air stream by using a glass cartridge with 50 $\mu$l $TiCl_4$. The distribution of smoke was approximately homogeneous in the arms of the Y-tube. Turbulent odour eddies, i.e. odour clouds and filaments, emerged in the stem of the Y-tube where the two air streams clash together.

*Evaluation*

The percentage of mosquitoes outside the release chamber after 30 s of stimulation was taken as the measure of upwind flight activity. The percentage of mosquitoes trapped in the test- and the control chamber, respectively, was taken as a measure for the attractiveness of the respective stimulus. For each stimulus the mean values (± S.E.) were calculated. Since the data are percentage values they were transformed using angle transformation (Sokal & Rohlf, 1981) for further statistical analysis. To compare the attractiveness of the stimulus side with the control side, a *t*-test for paired samples was used. For comparison of different stimuli, the values for flight activity and

*Table 1.* Arrangement of stimuli tested in different experiments. Method of stimulation (see Material and methods) is indicated by gc (= glass cartridge) and fp (= filter paper). $n$ = number of tested mosquito groups; each group with 20 mosquitoes

| | Treatment | $n$ | Stimulus in test-tube | Stimulus in control-tube |
|---|---|---|---|---|
| Experiment 1 | 1 | 10 | hand | empty filter paper (fp) |
| | 2 | 10 | empty filter paper (fp) | empty filter paper (fp) |
| Experiment 2 | 1 | 10 | 1 ml blank extract (fp) | 1 ml solvent (fp) |
| | 2 | 10 | 1 ml skin extract:100%vol. (fp) | 1 ml solvent (fp) |
| | 3 | 10 | 1 ml skin extract: 50%vol. (fp) | 1 ml solvent (fp) |
| | 4 | 10 | 1 ml skin extract: 25%vol. (fp) | 1 ml solvent (fp) |
| | 5 | 10 | 1 ml skin extract: 2.5%vol. (fp) | 1 ml solvent (fp) |
| Experiment 3 | 1 | 8 | 0.01 ml blank extract (gc) | 0.01 ml solvent (gc) |
| | 2 | 8 | 0.0001 ml skin extract (gc) | 0.01 ml solvent (gc) |
| | 3 | 8 | 0.0005 ml skin extract (gc) | 0.01 ml solvent (gc) |
| | 4 | 8 | 0.001 ml skin extract (gc) | 0.01 ml solvent (gc) |
| | 5 | 8 | 0.005 ml skin extract (gc) | 0.01 ml solvent (gc) |
| | 6 | 8 | 0.007 ml skin extract (gc) | 0.01 ml solvent (gc) |
| | 7 | 8 | 0.01 ml skin extract (gc) | 0.01 ml solvent (gc) |
| | 8 | 8 | 0.05 ml skin extract (gc) | 0.01 ml solvent (gc) |
| Experiment 4 | 1 | 10 | hand | empty glass cartridge (gc) |
| | 2 | 10 | 0.05 ml skin extract (gc) | 0.05 ml blank extract (gc) |
| | 3 | 10 | 0.05 ml skin extract (gc) | hand |
| Experiment 5 | 1 | 10 | 0.01 ml blank extract (gc) | 0.01 ml solvent (gc) |
| | 2 | 10 | 0.01 ml lactic acid solution (gc) | 0.01 ml solvent (gc) |
| | 3 | 10 | 0.01 ml skin extract (gc) | 0.01 ml blank extract (gc) |
| Experiment 6 | 1 | 10 | 0.01 ml lactic acid solution (gc) | 0.01 ml solvent (gc) |
| | 2 | 10 | 0.01 ml solvent (gc) + $CO_2$ | 0.01 ml solvent (gc) |
| | 3 | 10 | 0.01 ml lactic acid solution (gc) + $CO_2$ | 0.01 ml solvent (gc) |

the ones for attractiveness of the stimulus side were analysed independently using an One-way ANOVA followed by the Tukey–Kramer HSD *post hoc* test. Dose-response curves were calculated using a Probit analysis (Unkelbach & Wolf, 1985) and a non-linear regression analysis based on the sigmoid logistic function: $f = (A-B)/[1+(x/C)^P]+B$. $A$ represents the starting value (= threshold), $B$ the maximal reaction strength (= saturation level of the curve), $P$ describes the slope of the curve, and $C$ gives the dose, which elicited 50% of the maximum response (=ED50). The parameters were estimated by using the Levenberg-Marquard Method. The statistics were calculated with the program SPSS 6.0 for Windows, the dose response curves with Origin 4.1 Microcal.

*Determination of the solid content, lactate content, and the pH of the skin extract*

In order to determine the solid content of the skin extract, 1 ml of the skin extract was placed in a glass container and nitrogen was blown over it until its weight remained constant for 4 h. This was already obtained after 40 min. The lactate content was determined enzymatically with lactic dehydrogenase from rabbit muscle, (LDH, 550 U/mg, Boehringer, Germany) (Hohorst, 1970). Total lactate, i.e. both, salt and free acid, was measured as lactate at a pH of 9, since free L-(+)-lactic acid (pK value: 3.862 according to Rauen, 1964) is converted to lactate under these conditions. The pH-value of the skin extract was determined by using pH indicator paper (Merck, Germany). From this, the amount of free L-lactic acid in the skin extract was calculated.

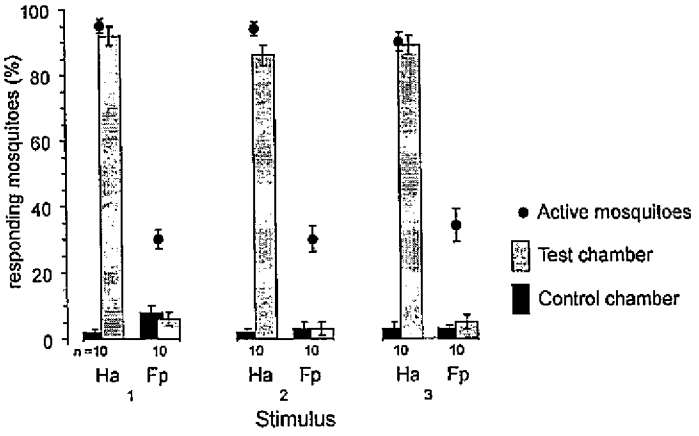


*Figure 3.* Responses of *Ae. aegypti* to a human hand. Columns show the mean percentage of the mosquitoes attracted to the test and the control chamber, respectively. Dots indicate the mean percentages (± S.E.) of the mosquitoes that had flown out the release chamber. On three different testing days (1, 2, 3) the behavioural responses of the mosquitoes to following stimuli were measured: hand of test person (Ha), clean filter paper (Fp).

## Results

*Responses to a human hand.* On three different days the hand consistently yielded very high values of flight activity and attractiveness (90%–95% and 86%–92%, respectively). Figure 3 shows the responses to the hand and to an empty filter paper. The differences between the test and the control chambers were always highly significant ($P < 0.001$; $t = 12.76$, 17.63, and 19.01; df 9; $t$-test for paired samples). The behavioural responses to the hand, as well as to the controls, did not differ significantly between the different days (one-way ANOVA, $P > 0.05$, for flight activity as well as attractiveness values of test and control chamber, respectively). Most of the mosquitoes left the release chamber just a few seconds after stimulation and flew upwind. They then moved towards the test side of the Y-tube and landed on the gauze screen which was located directly in front of the hand. There, some ran back and forth and repeatedly probed through the screen or flew to another place on the screen. No mosquito was observed to fly back to the release chamber. When a clean filter paper was used instead of a hand, only a small percentage (3% and 8%) of mosquitoes was found in the test and the control chamber, respectively and there was no significant preference for a particular chamber ($P_1 = 0.252$, $t_1 = -1.22$; $P_2 = 0.642$, $t_2 = 0.48$; and $P_3 = 0.397$, $t_3 = 0.89$: df 9 each, $t$-test for paired samples). In these control experiments the number of activated mosquitoes was significantly lower than with stimulation by the hand ($P<0.0001$, $F[5:54]=70.12$, one-way ANOVA; Tukey–Kramer HSD: $P<0.05$). The mosquitoes never attempted to probe through the gauze of either the test or the control chamber.

*Responses to the skin extract.* In order to determine the effectiveness of the skin extract, the dose response relationship was measured with heated glass cartridges as well as with unheated filter papers as stimulus sources. Figure 4 shows the results for both stimulation methods. The commonly used probit model in pharmacological dose-response relationships (Unkelbach & Wolf, 1985), did not yield an adequate curve fit ($\chi^2$ model adaptation test: $T = 19.85$, df $= 4$, $P = 0.001$), whereas a sigmoidal logistic function of a non-linear regression analysis yielded an acceptable fit (Figure 4). The curve for the filter papers is determined by the parameters $A = 11.0$, $B = 100.0$, $c = 294.4$, and $p = 1.5$, whereas the curve for the glass cartridges is determined by the parameters $A = 4.0$, $B = 88.6$, $c = 0.8$, and $p = 1.4$. With both methods of stimulus delivery the maximum percentage of attracted mosquitoes was around 90%, a value which was also attained in previous experiments with the human hand. To directly compare the effect of both stimuli, 50 $\mu$l skin extract from one person and the hand of the same person were tested separately as well as simultaneously (Figure 5). In the first case the attractiveness and the flight activity for stimulation with the hand and the skin extract, respectively, were not significantly different (flight activity: $P = 0.207$, $t = 1.31$, df $= 18$;

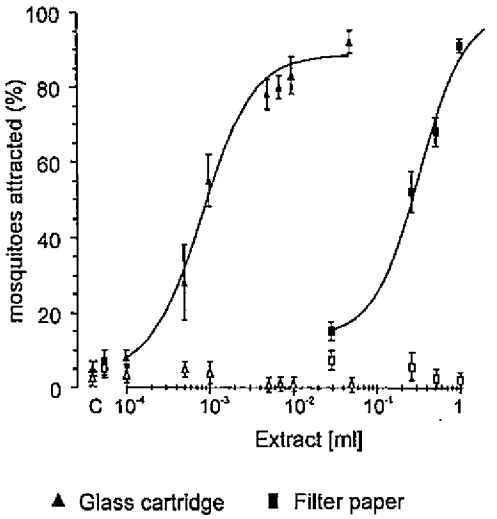


*Figure 4.* Dose-response curves of responses of *Ae. aegypti* to skin extract. Rectangular symbols represent the mean percentages (± S.E., $n = 8$) of tests with heated glass cartridges as stimulus source, triangular symbols the mean percentages (± S.E., $n = 10$) of tests with unheated filter papers as stimulus source. Filled symbols stand for mean percentages of the test side, empty symbols for the mean percentages of the control side. X-axis represents the amount of skin extract in $\mu$l in the glass cartridge or on the filter paper, respectively. $C$ = control treatments with blank extract (10 $\mu$l on glass cartridge and 1 ml on filter paper, respectively). The curve fittings yielded from regression analysis using the sigmoidal logistic function: $f = a \cdot (x/c)^b/[(x/c)^b + 1]$.

attractiveness test chamber: P = 0.447, $t = 0.78$, df = 18; $t$-test for unpaired samples). In the direct comparison the mosquitoes were evenly distributed in both chambers at the upwind side (P = 0.991, $t = 0.01$, df = 9; $t$-test for paired samples), which again indicates that both stimuli were equally attractive.

*Responses to L-(+)-lactic acid and carbon dioxide.* Since L-(+)-lactic acid has been shown an attractive component of acetone washes from human skin (Acree, 1968), we determined the amount of L-(+)-lactic acid in our skin extract. Using an enzymatic lactate test, a concentration of 11 mg/ml lactate was determined (total solids content: 67 mg/ml). The pH was approx. 5. Given that L-(+)-lactic acid has a pK value of 3.862 we calculated that 7% of the total lactate was free L-(+)-lactic acid (i.e. a concentration of 0.8 mg/ml free lactate acid). To compare the effect of lactic acid with the complete skin extract the following stimuli were tested: (1) blank extract, (2) skin extract, and (3) an equivalent amount of L-(+)-lactic acid. The results are shown in Figure 6. L-(+)-lactic acid activated more mosquitoes (P<0.001, F[2:27]=31.96:, One-way ANOVA; Tukey–Kramer HSD: P<0.05) than the blank extract and was significantly attractive (P<0.001, $t = 8,17$, df = 9, $t$-test for paired samples) in contrast to the blank extract (P = 0.077, $t = 2.0$, df = 9; $t$-test for paired samples). However, the skin extract was considerably more attractive than an equivalent L-(+)-lactic acid stimulus (P<0.001, F[2:27]=62.79, One-way ANOVA; Tukey–Kramer HSD: P<0.05).

In an additional experiment we investigated the effects of carbon dioxide alone and in combination with L-(+)-lactic acid. The following stimuli were tested: (1) solvent, (2) carbon dioxide, (3) L-(+)-lactic acid, and (4) a combination of carbon dioxide and L-(+)-lactic acid. Carbon dioxide activated significantly more mosquitoes than L-(+)-lactic acid (P<0.001, F[2:27]=23.76, one-way ANOVA; Tukey–Kramer HSD: P<0.05) and was also more attractive (P<0.001, F[2:27]=37.17, one-way ANOVA; Tukey–Kramer HSD: P<0.05) (Figure 7). Furthermore, the attraction to the combined stimuli (86%) was higher than the mere added responses to the single stimuli (61%), indicating a synergistic effect. The combined stimuli also increased the flight activity, but not in a synergistic manner (Figure 7).

Even after multiple tests with the same mosquitoes during a day, the behavioural responses to L-lactic acid were not changed remarkably. Pooling the data of many tests, the mean percentage of attracted mosquitoes which were used for the first time, was 28% S.E. = 2.1, and the mean percentage of activated mosquitoes was 61% S.E. = 2.4 ($n = 48$ mosquito groups). After these mosquitoes had been tested 6–10 times with various stimuli (control, hand, carbon dioxide, or lactic acid), 24% S.E. = 2.3 were attracted to lactic acid and 64% S.E. = 2.8 were activated ($n = 48$ groups) by this compound.

In order to find out possible contamination control runs without odour were performed directly after stimulation with the hand or lactic acid, respectively. Table 2 shows that previous presentation of such stimuli in an arm of the Y-tube did not result in a higher attraction to this arm or in an increase of flight activity in successive control tests.

## Discussion

In the described Y-tube odour stimuli like carbon dioxide, lactic acid, and human skin residues attracted

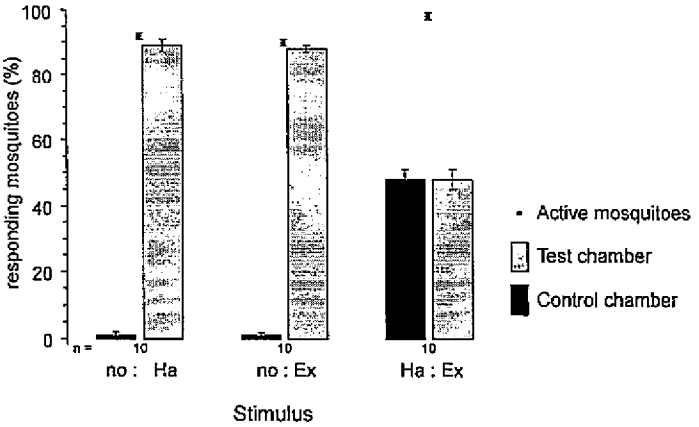


*Figure 5.* Comparison of responses of *Ae. aegypti* to hand and skin extract. Columns show the mean percentage (± S.E.) of the mosquitoes attracted to the test and the control chamber, respectively. Circles indicate the mean percentages (± S.E.) of the mosquitoes that had flown out of the release chamber. Stimuli tested: hand in the test chamber (Ha), no stimulus in the control chamber (no); 50 µl skin extract in the test chamber (Ex), no stimulus in the control chamber (no); 50 µl extract in the test chamber (Ex), hand in the control chamber (Ha).

*Table 2.* Behavioural responses of *Ae. aegypti* as an indication for possible contamination effects after previous treatments with odour stimuli. Mean percentages (±S.E.) of mosquitoes found in the test chamber[a], control chamber[b], and outside the start chamber[c] in tests with the mentioned odour treatments, and in subsequent control tests without odour stimulation. The data of *n* tests (20 mosquitoes per test) from different experiments were pooled

| Previous stimulus (*n*) | Previous tests | | | Subsequent control tests | | |
|---|---|---|---|---|---|---|
| | Test[a] | Control[b] | Activity[c] | Previous test[a] | Previous control[b] | Activity[c] |
| Hand (10) | 81 (3.7)* | 2 (1.1) | 93 (1.9)a | 7 (1.5) NS | 3 (1.3) | 39 (5.1)a |
| Skin extract (10) | 73 (3.2)* | 3 (1.3) | 89 (3.2)a | 6 (1.5) NS | 2 (1.1) | 41 (4.2)a |
| Lactic acid (9) | 23 (4.2)* | 5 (1.4) | 53 (5.6)b | 3 (1.2) NS | 6 (2.6) | 35 (5.5)a |
| No odour (8) | 6 (1.8)NS | 11 (2.9) | 34 (5.0)c | 3 (1.3) NS | 5 (2.7) | 35 (5.7)a |

*Significantly attractive compared to the control chamber (*t*-test, $P<0.05$; NS= non significant). Means of the activity values in one column followed by same letter do not differ significantly (ANOVA, $P<0.05$, Tukey–Kramer HSD).

female yellow fever mosquitoes *Ae. aegypti* within 30 s towards the stimulus outlet or source. The percentage of responding mosquitoes depended upon the quality as well as on the dose of the stimuli. Carbon dioxide stimulated the upwind flight activity and was significantly attractive, but considerably fewer mosquitoes (ca. 40%) were attracted by this compound than by a human hand (ca. 90%). Addition of lactic acid increased the attractiveness of carbon dioxide in a synergistic manner. The reactions to both compounds correspond closely to those described by other authors (Eiras & Jepson, 1991; Gillies, 1980; Takken, 1991; Davis & Bowen, 1994; Bowen, 1991; Sutcliffe, 1987; Acree, 1968; Smith et al., 1970; Carlson et al., 1973). Acree et al. (1968), Smith et al. (1970), and Eiras & Jepson (1991, 1994) found lactic acid attractive only in combination with carbon dioxide concentrations above atmospheric level. In our experiments, however, lactic acid was significantly attractive without an increase of the concentration of carbon dioxide in the olfactometer air. We do not entirely exclude the possibility that the mosquitoes from our culture might have responded more sensitive to lactic acid than the ones used in other investigations. Mukwaya (1977) found indeed differences in the host finding behaviour of different cultures of the same mosquito species. However, preliminary tests with offsprings from a culture of *Ae. aegypti* from the Swiss Tropical Institute revealed also a significant attractiveness of lactic acid alone. Our experiments were performed at

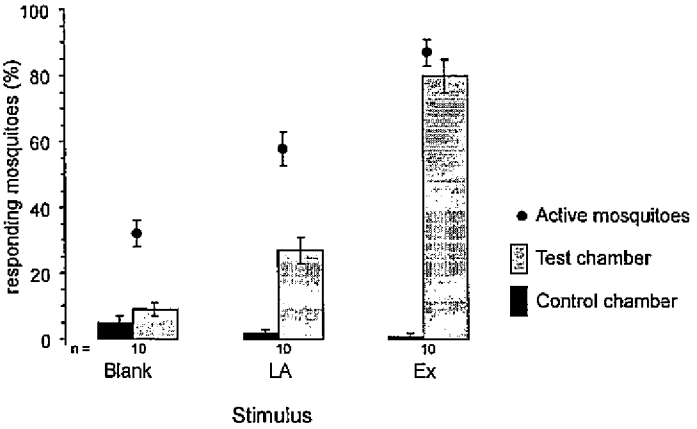


*Figure 6.* Comparison of responses of *Ae. aegypti* to skin extract and L-(+)-lactic acid. Columns show the mean percentage (± S.E.) of the mosquitoes attracted to the test and the control chamber, respectively. Circles indicate the mean percentages (± S.E.) of the mosquitoes that had flown out of the release chamber. Stimuli tested: glass cartridge with 10 $\mu$l blank extract (Blank); glass cartridge with 8 $\mu$g L-(+)-lactic acid (LA); glass cartridge with 10 $\mu$l skin extract containing 8 $\mu$g L-(+)-lactic acid (Ex).

similar temperature, humidity, and wind speed as were present in other investigations, and slight variations of these parameters do not seem to effect the responses of the mosquitoes (Rössler, 1961). Our choice to use only mosquitoes, which had been lured by the human hand, cannot be the reason for the attractive effect of lactic acid in our study. Other authors (Eiras & Jepson, 1991; Smith et al., 1970), who did not observe a significant response to lactic acid, selected the mosquitoes in the same way. The fact that we observed an attractive effect of lactic acid alone might be due to certain geometrical properties of the Y-tube, to the special mode of stimulus presentation, or to a certain the distribution of the odours in the air stream. The fast responses of the mosquitoes and the strong attraction to a human hand or to the skin extract support this interpretation.

The design of the Y-tube olfactometer permits the use of the human hand as a source of natural host odour. Considering the fact that the moisture and warmth given off by a human hand may enhance the attractiveness of the skin odours (Davis & Bowen, 1994), the hand represents the most natural set of attractive stimuli. According to Miller & Strickler (1984) such a natural stimulus source is important for a critical evaluation of the effect of attractants. Since the mean attractiveness of the hand of one test person averaged over one day remained constant over 2 months, this stimulus source seems to be a simple reference to assess the effect of sampled odoriferous material from human skin. In the past host odours from different sources like human perspiration (Parker, 1948; Rössler, 1961; Skinner, et al., 1965, 1968; Müller, 1968; Eiras & Jepson, 1991), urine (Rössler, 1961), blood (Rudolfs, 1922; Schaerffenberg & Kupka, 1959; Brown & Carmichael, 1961), skin washings (Acree et al., 1968; Smith et al., 1970; Schreck et al., 1981, 1990), and headspace samples from humans (Bar-Zeev et al., 1977) or mice (McCall et al., 1996) were demonstrated to be attractive for mosquitoes, but in all these studies the odoriferous material was never compared with the reference of the natural host from which the material was sampled. Our results show that the odour from skin residues of an attractive person attracts yellow fever mosquitoes almost as well as the hand of this person. This indicates that the extract contains at least the most important kairomones. Threshold, saturation level, slope, and the ED 50 value of the sigmoidal dose-response curve characterise the extract's effectiveness; by these parameters, extracts from other sources (e.g., either from different human test subjects or different host species) can be compared in future.

To present such an extract in the bioassay, we tested two different methods: (1) the odourants were blown into the olfactometer from a heated glass cartridge (60 °C) coated with extract, and (2) a non heated filter paper loaded with extract was placed directly in the air stream of the olfactometer. The comparison of the according dose-response curves show that for similar behavioural responses a more than 300 fold higher dose of extract is required on filter papers than

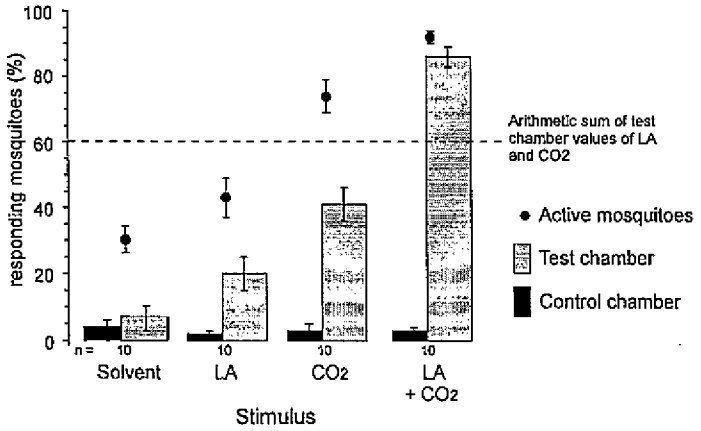


*Figure 7.* Synergis tic effect of L-(+)-lactic acid and carbon dioxide. Columns show the mean percentage (± S.E.) of the mosquitoes attracted to the test and the control chamber. Circles indicate the mean percentages (± S.E.) of the mosquitoes that had flown out of the release chamber. Stimuli tested: glass cartridge with 10 $\mu$l ethanol (Solvent); glass cartridge with 8 $\mu$g L-(+)-lactic acid (LA); 0.1% $CO_2$ in the test chamber ($CO_2$); simultaneous introduction of these stimuli (LA + $CO_2$).

in heated glass cartridges (ED $50_{\text{glass cartridge}}$: 0.8 $\mu$l, ED $50_{\text{filter paper}}$: 250 $\mu$l). Mosquitoes are known to respond sensitively to temperature fluctuations (Davis & Bowen, 1994). Nevertheless, the heat stimulus which is given off together with the odour from the glass cartridges is not a requirement for strong behavioural responses to host odours. The results with non-heated filter papers as odour source indicate that the same responses can be evoked by the odour stimulus alone. Moreover, the heat stimulus of an empty glass cartridge was never attractive. By heating the extract, the output of attractive compounds is increased by either an increase of evaporation of odour components or by acceleration of chemical reactions and decomposition. This is probably the main effect of heating the stimulus source, although it is also possible that the heat stimulus synergizes the effect of an attractive odour. The principal advantage of heating is the reduced consumption of test material. In this way, the bioassay becomes a more sensitive tool for chemical analysis to detect and identify attractive components.

Schreck et al. (1981, 1990) reported strong contamination effects caused by touching the test equipment with hands. We also observed these effects shortly after construction of the olfactometer. Once the apparatus was thoroughly cleaned with detergents, hot water, and ethanol we did not observe any contamination effects provided neither test substances nor human skin came directly into contact with the inside of the stimulus tubes. This indicates that contamination is probably caused by vestiges of solid or fluid material adhered to surfaces rather than by adsorption processes in the gaseous phase. We gauged our procedure by repeatedly performing control experiments and found no hints for contamination effects created during the test program.

The bioassay used in these experiments has a relatively high testing capacity (six behavioural tests can be performed in one hour using one olfactometer). It allows sensitive measurements of attraction to odours, and provides for a comparison with the natural stimulus. For this reason, it appears to be well suited for routine tests of various odour samples and as a tool for the identification of attractive components in these samples. It is clear that besides L-(+)-lactic acid, other compounds contribute to the attractiveness of the human skin residues. Further identification of these compounds will be a matter of chemical analysis in close interaction and co-operation with biological testing.

## Acknowledgements

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# Ammonia as an Attractive Component of Host Odour for the Yellow Fever Mosquito, *Aedes aegypti*

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## Abstract

Behavioural responses of *Aedes aegypti* mosquitoes to ammonia were investigated in a modified Y-tube olfactometer. Ammonia was attractive in concentrations from 17 ppb to 17 ppm in air when presented together with lactic acid. Aqueous solutions of ammonia salts in concentrations comparable to those found in human sweat also increased the attractiveness of lactic acid. The role of lactic acid as an essential synergist for ammonia became further apparent by the fact that ammonia alone or in combination with carbon dioxide was not effective, even though the synergistic effect of carbon dioxide and lactic acid was corroborated. An extract from human skin residues, which attracts ~80% of the tested mosquitoes, contains both lactic acid and ammonia. The combination of these compounds, however, attracts no more than 45%, indicating that other components on human skin also play a role in host finding. Preparative liquid chromatography of the skin extract yielded three behaviourally active fractions which work together synergistically. Fraction III contains lactic acid as the effective principle; the compositions of the other two have not been clarified yet. The attractiveness of fraction I was augmented considerably when ammonia was added, whereas the effect of fraction II was not influenced by ammonia. These results suggests that ammonia is part of the effective principle of fraction II and contributes to the attractive effect of host odours.

## Introduction

Olfactory cues are widely used by bloodsucking insects to detect and to find their sources for blood meals. Since mosquitoes are one of the most important groups of vectors for human and animal disease, many attempts have been undertaken to explore the attractive blend of host odours. Different mosquito species develop different host preferences, and it is generally assumed that host selection and discrimination is mainly based on olfactory cues (Takken, 1991). Until today, however, only a few attractive components of host odour have been identified and we know little about the role of these volatiles in the complex network of behavioural sequences which lead these insects to their warm blooded hosts. Almost all mosquito species use carbon dioxide, which is given off from hosts with breath, as an alerting and attractive signal. L-(+)-Lactic acid, a major component in breath and on human skin, attracts *Aedes aegypti* in that it acts as an essential synergist when combined with carbon dioxide as well as with volatiles from the skin (Acree, 1968; Smith *et al.*, 1970; Eiras and Jepson, 1991; Geier *et al.*, 1996). Recently, a synthetic mixture of 12 aliphatic fatty acids, identified in the head space of Limburger cheese, has been implicated as attractive for *Anopheles gambiae* (Knols *et al.*, 1997). In the past, certain amines, oestrogens, amino acids and alcohols have also been reported to attract mosquitoes, but many of these results were contradictory and synthetic odour blends never matched the effect of the natural host odour (Hocking, 1971; Takken, 1991). Previous studies in our laboratory with yellow fever mosquitoes, *Ae. aegypti*, have revealed that other components besides lactic acid contribute to the high attractiveness of human skin residues (Geier *et al.*, 1996). Interestingly, the components were only attractive in combination with lactic acid. These findings indicate that attractive effects of certain compounds can be discovered in a bioassay only in combination with lactic acid. A promising candidate of such an attractant is ammonia, since this compound has been identified in effluvia from vertebrates (Larson *et al.*, 1979; Norwood *et al.*, 1992). Attractive effects of ammonia have already been reported for a range of haematophagous arthropods (Taneja and Guerin, 1997; Hribar *et al.*, 1992). Müller (Müller, 1968), Brown (Brown, 1952) and Rössler (Rössler, 1961) could not find any attractive effect of ammonia in behavioural experiments with *Ae. aegypti*, but they never tested this compound in combination with lactic acid. Taking the synergistic effects of lactic acid and also of carbon dioxide into account, we reinvestigated whether ammonia could be an attractant also for *Ae. aegypti*. In a modified Y-tube olfactometer we tested

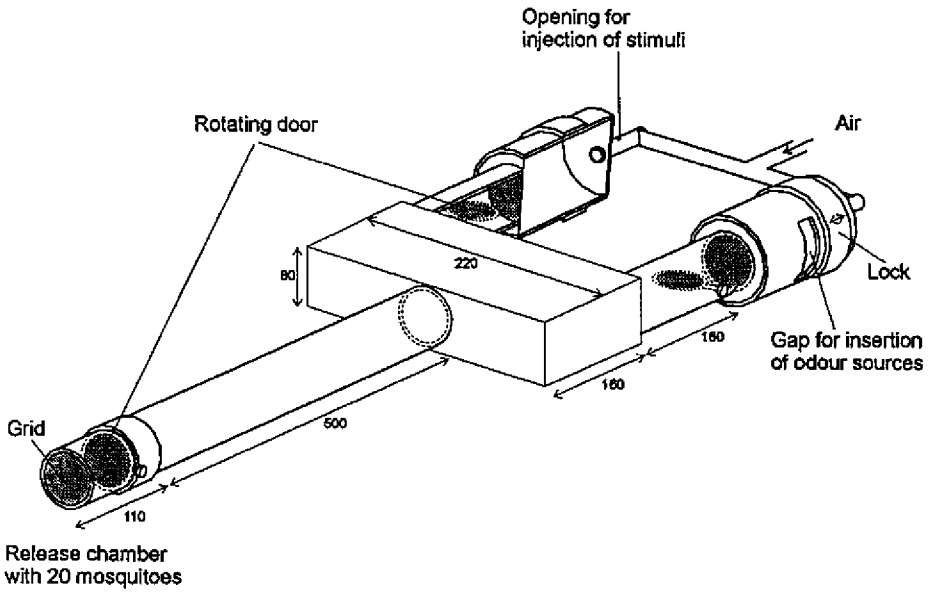


**Figure 1** Schematic drawing of the olfactometer. Dimensions are given in mm.

the attractiveness of different sources of ammonia over a wide range of concentrations alone and in different combinations with lactic acid, carbon dioxide and behaviourally active fractions of an extract from human skin residues. Such an extract has been shown to attract *Ae. aegypti* and was separated by means of liquid chromatography in three behaviourally active parts: fractions I and II do not contain lactic acid and have no attractive effect on their own, but they act synergistically together with fraction III, which contains lactic acid as the effective principle (Geier *et al.*, 1996). We tested whether ammonia enhances the effect of one of these fractions.

## Materials and methods

### Insects

Female *Ae. aegypti*, 5–15 days old, from cultures of the centre for Plant Research at Bayer AG in Monheim, were used in our experiments. The larvae were fed with Tetramin® fish food. Some 300–500 adults were kept in containers (50 × 40 × 25 cm) at 26–28°C and 60–70% relative humidity, with a 12 h:12 h L:D photoperiod. They had access to a 10% glucose solution on filter paper. Since male and females were kept together, we presume that all females had been mated before they were used in the experiments. Shortly before the experiments, we lured the mosquitoes out of their containers by means of the human hand as bait. This ensured that the tested insects were able to respond to the host.

### Olfactometer

A modified Y-tube olfactometer (Geier, 1995) was used to measure the attractiveness of odours (Figure 1). The branch of the Y-tube consisted of a rectangular Plexiglas chamber, in which the two arms run into one side and the stem runs into the opposite side (Figure 1). Each of both arms fitted into a PVC® stimulus chamber where the odours were mixed with the air flushing the olfactometer. The release chamber with the mosquitoes was attached to the downwind end of the stem. Rotating screens in the release chamber as well as in both arms at the downwind end permit the release or the entrapment of the mosquitoes respectively. A constant airstream (flow rate: 80 l/min) from the institute's pressurized air system was purified by a filter of activated charcoal, heated and humidified before passing through the olfactometer. Further details of this experimental arrangement are described elsewhere (Geier, 1995). The temperature was 28 ± 1°C, the relative humidity 70 ± 5% and the wind speed 0.2 m/s in the arms and 0.4 m/s in the stem respectively. The olfactometer was placed on a white table with white cardboard shields (height: 20 cm) on both sides to prevent visual stimulation by the experimenter. The room was illuminated by two 40 W light bulbs.

### Odour stimuli and stimulus delivery

Three different sources of ammonia stimuli were tested. To measure the dose–response curve (experiment 1) different amounts of ammonia were produced according to the procedure of Ough and Stone (Ough and Stone, 1961). Charcoal-filtered air at flow rates of 0.03–300 ml/min was passed through an Erlenmeyer flask filled with 50 ml of an aqueous solution of 0.13 mmol/l $NH_3$ (p.A., Merck, Darmstadt, Germany) in distilled water. Charcoal-filtered air at a flow rate of 300 ml/min passed through an Erlenmeyer flask filled with 50 ml distilled water served as a control. The air was passed over the surface of the solutions. To determine

the output of $NH_3$, the air of four different flow rates, 0.3, 3, 30 and 300 ml/min, was trapped in gas wash flasks with a solution of 0.01 N HCl for 23 h, 1 h, 30 min and 12 min respectively. At the highest flow rate the trapping flasks were filled with 50 ml of the HCl solution; at the lower flow rates they were filled with 10 ml. We connected up to four trapping flasks in series to monitor any breakthrough from a previous one. The amount of ammonia was determined by titration with 0.01 N NaOH solution (Poethke, 1987). Figure 2 shows the relationship between the flow rate through the stimulus source and the total amount of $NH_3$ trapped per minute. In experiments 2, 3 and 5 the ammonia was delivered in the same way with a flow rate of 3 ml/min, which resulted in an output of 5 µg/min and a concentration of 7 nmol/l air (170 ppb) in the test chamber of the olfactometer.

In experiment 4 we tested an aqueous solution of 10 mmol/l ammonium chloride (p.A., Merck, Darmstadt, Germany) in distilled water. The pH value of this solution, determined by means of a pH indicator paper (Merck), was found to be 6.5. Small open glass vials (height: 30 mm; inner diam.: 16 mm) were filled with 2 ml of this solution and placed into the stimulus chamber. A 10 mmol/l NaCl solution served as control. In this experiment a L-(+)-lactic acid/ammonia buffer which simulates the composition of human sweet was also tested. For this, 400 mg of L-(+)-lactic acid (p.A., Merck) and 270 mg of 25% ammonia were dissolved in 50 ml of distilled water. This resulted in a lactate/ammonium salt solution of 89 mmol/l lactic acid and 80 mmol/l ammonia with a pH value of 5 and a surplus of free lactic acid; 0.5 ml of this solution was applied to filter paper discs (7 cm diam.) and the wet discs were put into the stimulus chamber. A lactic acid/sodium lactate buffer with 450 mg of L-(+)-sodium lactate (p.A. Merck) and 40 mg of L-(+)-lactic acid dissolved in 50 ml of distilled water served as a control. The pH value of this solution (80 mmol/l sodium lactate and 9 mmol/l lactic acid) was also 5, indicating the same amount of free lactic acid as in the lactate/ammonium salt solution.

Lactic acid stimuli were generated using a set-up similar to one based on the design of Ough and Stone (Ough and Stone, 1961), described above. Charcoal-filtered compressed air at a flow rate of 15 ml/min was passed through a 250 ml Erlenmeyer flask filled with 10 ml of L-(+)-lactic acid solution (90% in aq. sol.; Merck, Darmstadt, Germany). According to the calibration of Geier *et al.* (Geier *et al.*, 1999), at this flow rate an output of 3 µg/min lactic acid was generated and led into the stimulus chamber. This dose is in the range of the lactic acid given off from human hands (0.4–2.22 µg/min) after data from Smith *et al.* (Smith *et al.*, 1970).

The carbon dioxide used in experiment 3 was taken from a gas cylinder having the trade-standard purity of 99.9% (Linde, Nürnberg, Germany). The gas was injected into the stimulus chamber at a flow rate of 1600 ml/min and homogeneously mixed with the olfactometer air, yielding a concentration of 4% in the test arm of the olfactometer.

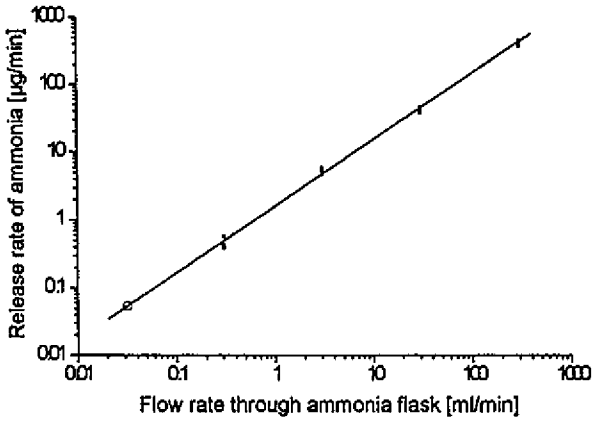


**Figure 2** Calibration curve of ammonia output from an Erlenmeyer flask filled with 50 ml of an aqueous solution of 0.13 mmol/l $NH_3$ in distilled water. Each dot shows the amount of ammonia trapped in solutions of HCl at flow rates between 0.3 and 300 ml/min. Since we used these flow rates in the bioassays, the amount of ammonia released at a flow rate of 0.03 ml/min was extrapolated.

A skin extract was obtained according to a method described in detail by Geier *et al.* (Geier *et al.*, 1996). Hands, forearms, feet and calves were rubbed for 5 min with pads, which were then extracted with methanol (p.A. Fluka, Germany). The extracts from 50 pads sampled within a period of 2 months were combined, concentrated to 30 ml by evaporation in a rotary evaporator, and then centrifuged at –20°C (950 *g*; 2 h) to yield a clear yellow supernatant extract with a concentration of free L-(+)-lactic acid of 6 mmol/l (Geier *et al.*, 1996). The $NH_3$ concentration was 7.4 mmol/l, measured using a gas-sensitive $NH_3$ electrode after the method of standard addition in the research laboratory of the Bayer AG (Camman, 1979).

The skin extract was fractionated on a preparative silicagel column with acetonitrile and ethanol, yielding three separate fractions—fractions I, II and III (Geier *et al.*, 1996). A blank extract made from 50 cotton pads served as a control. For stimulus delivery a volume of 0.01 ml of skin extract or fractions, respectively, were applied to the inner side of a glass cartridge (inner diam.: 3.3 mm; length: 5 cm). After the solvent had evaporated, the glass cartridge was placed into a heating element on top of the stimulus chamber and air was blown through it at a rate of 2.8 l/min to deliver the odours from the surface of the glass cartridge as described elsewhere (Geier and Boeckh, 1999). The flow rate of the airstream was regulated and controlled by flow meters (Rota GmbH, Germany).

### Odour distribution

Since we know that the spatial distribution of odours influences the attractiveness of odour sources (Geier *et al.*, 1999), we visualized the distribution of the odorants by

means of $TiCL_4$ smoke. In the arms of the olfactometer smoke was equally distributed similar to the homogeneous plume type outlined by Geier *et al.* (Geier *et al.*, 1999). More turbulent odour eddies, i.e. odour clouds and filaments, emerged in the rectangular Plexiglas chamber and the stem of the Y-tube respectively, where the two airstreams of the arms come together.

### Bioassay

Bioassays were conducted as described in detail elsewhere (Geier *et al.*, 1999). Groups of 18–22 female mosquitoes were used for the tests. Before stimulation, the mosquitoes were given 20 min to acclimatize. Between the tests a constant flow of fresh air flushed the olfactometer; the bioassays ran from 9:00 a.m. to 6:00 p.m. The odour stimuli were tested in five blocks of tests, in which the stimuli were tested repeatedly in random order. For each block of experiments a different mosquito population was used.

### Evaluation of activation and attraction

In each test we distinguished two behavioural categories of responses: (i) the percentage of mosquitoes found outside the release chamber after 30 s was taken as a measure for activation, which included taking flight and short upwind progress. (ii) The percentages of mosquitoes trapped at the upwind end of the test- and control chambers, respectively, were taken as measures for attractiveness of the test- and control odours. For each stimulus the means (± SE) of activation and attractiveness were calculated. Since the data are percentage values, they were transformed using angle transformation (Sokal and Rohlf, 1981) for further statistical analysis. The transformed means were analysed independently by a one-way ANOVA using the LSD method as a *post hoc* test for comparison of the treatments. All calculations and statistics were performed with the statistics program SPSS 8.0 for Windows.

## Results

Lactic acid at the dose of 3 µg/min, which is in the range of evaporation rates from human hands (Smith *et al.*, 1970), attracted ~20% of the mosquitoes (Figure 3). The attractiveness of this stimulus was significantly increased by addition of ammonia over a wide range of concentrations (Figure 3). The effective concentration range of ammonia was between 0.7 and 700 nmol/l air (17 ppb–17 ppm). The lowest ammonia concentration of 0.07 nmol/l (1.7 ppb) did not affect the response to the lactic acid stimulus, indicating a threshold between 0.07 and 0.7 nmol/l air (1.7–17 ppb). Addition of 7 nmol/l air (170 ppb) ammonia doubled the percentage of mosquitoes attracted to lactic acid alone. Higher concentrations did not further raise the attractiveness. From 0.7 to 70 nmol/l air (17–1700 ppb) ammonia alone was not attractive compared with controls.

In a direct choice situation, when ammonia and lactic

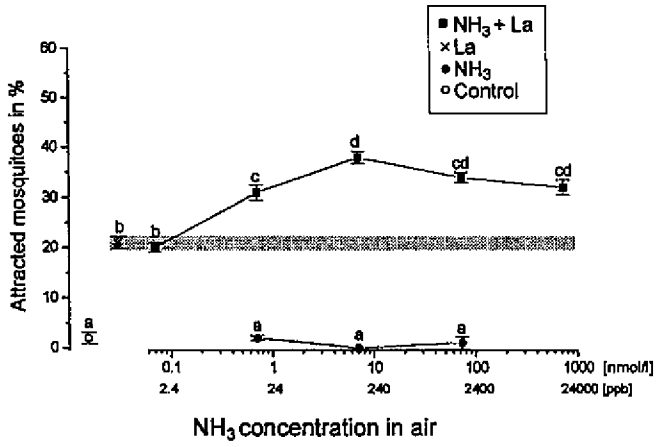


**Figure 3** Experiment 1: relationship of mean (± SE) percentages of mosquitoes trapped in the test chamber of the olfactometer and different concentrations of ammonia in the olfactometer air. The ammonia concentrations are calculated by the amounts released from the stimulus source and the flow rate of air flushing the test chamber. Distilled water served as the control stimulus. Each dot shows the mean of 28 tests, each using with 18–22 mosquitoes. Means were compared with each other using a one-way ANOVA and the LSD *post hoc* test; the letter code above the dots indicates significant differences: means with no letter in common are significantly different ($P < 0.05$).

acid were tested simultaneously as separate stimuli, all mosquitoes preferred significantly the lactic acid source (experiment 2, Table 1). A combination of lactic acid and ammonia, however, was significantly more attractive than lactic acid alone. When the stimulus in both chambers was lactic acid, both chambers were equally attractive.

In contrast to the synergism found between ammonia and lactic acid, no such effect was observed when ammonia was added to carbon dioxide (experiment 3, Table 1). A concentration of 4% carbon dioxide had a strong activating as well as a slight attractive effect upon the mosquitoes. By adding ammonia at a concentration of 7 nmol/l air (170 ppb) neither the percentage of activated mosquitoes nor the percentage of attracted mosquitoes was significantly higher than with carbon dioxide alone. In the same experiment, however, a combination of carbon dioxide and lactic acid attracted nearly 80% of the mosquitoes, indicating a strong synergistic effect between these stimuli.

In experiment 4 we tested two other ammonia sources, which mimic the composition of human sweat. The results summarized in Table 1 show that an aqueous solution of ammonium chloride increased the attractiveness of the lactic acid standard stimulus. In addition, the aqueous buffer system of lactic acid and ammonia attracted a significant higher percentage of mosquitoes than a buffer of lactate/lactic acid at the same pH.

Both fractions I and II, obtained by means of preparative liquid chromatography of a highly attractive skin extract, have been shown to increase the attractiveness of lactic acid, but they had no effect on their own (Geier *et al.*, 1996).

**Table 1** Behavioural responses of *Ae. aegypti* to ammonia stimuli

| Experiment | Test chamber | | | Control chamber | | | Active | |
|---|---|---|---|---|---|---|---|---|
| | Stimulus | % T[1] | SE | Stimulus | % C[2] | SE | % A[3] | SE |
| 2 | lactic acid + $NH_3$ | 25 | 4.8* | $NH_3$ | 8 | 2.1 | $66.9^a$ | 6.4 |
| | lactic acid | 14.6 | 2.7* | $NH_3$ | 0.4 | 0.4 | $63.3^a$ | 4.3 |
| | lactic acid | 10.4 | 1.6 | lactic acid | 10.9 | 2.4 | $57.6^a$ | 4.8 |
| 3 | $CO_2$ | $10^a$ | 1.7* | no | 0 | 0 | $91^a$ | 2.8 |
| | lactic acid + $CO_2$ | $79^b$ | 2.7* | no | 0 | 0 | $97^a$ | 1.3 |
| | $NH_3$ + $CO_2$ | $7^a$ | 2.1* | no | 0 | 0 | $86^a$ | 3.4 |
| 4 | solvent | $3^a$ | 1.6 | solvent | 1 | 2 | $35^a$ | 4.1 |
| | NaLa | $19^b$ | 2.7* | solvent | 2 | 3 | $69^{bc}$ | 2.8 |
| | AmLa | $39^c$ | 3.7* | solvent | 3 | 4 | $80^c$ | 5.2 |
| | lactic acid + NaCl | $22^b$ | 2.1* | solvent | 2 | 1.5 | $64^b$ | 2.1 |
| | lactic acid + AmCl | $44^c$ | 2.9* | solvent | 1 | 1.1 | $83.5^c$ | 2.3 |
| 5 | lactic acid | $20^a$ | 2* | solvent | 0 | 0 | $65^a$ | 2.2 |
| | lactic acid + FrI | $50^b$ | 3.6* | solvent | 0 | 0 | $84^b$ | 1.8 |
| | lactic acid + $NH_3$ + FrI | $67^c$ | 3.3* | solvent | 0 | 0 | $87^b$ | 2.3 |
| | lactic acid + FrII | $67^c$ | 3* | solvent | 0 | 0 | $85^b$ | 1.9 |
| | lactic acid + $NH_3$ + FrII | $65.8^c$ | 3.2* | solvent | 0 | 0 | $81^b$ | 2.2 |
| | lactic acid + FrI + FrII | $82^d$ | 1.9* | solvent | 0 | 0 | $90.2^b$ | 1.3 |
| | skin extract | $85^d$ | 2* | solvent | 0 | 0 | $94.2^b$ | 1.4 |

Means from 20 tests per treatment; each test with 18–22 mosquitoes. Abbreviations: NaLa = L-(+)-lactic acid/sodium lactate buffer, AmLa =L-(+)-lactic acid/ammonia buffer, AmCl = aqueous solution of ammoniumchloride, NaCl = aqueous solution of sodium chloride, FrI and FrII are two fractions of liquid chromatography separation of the skin extract.

[1]Mean percentage of mosquitoes trapped in the test chamber.

[2]Mean percentage of mosquitoes trapped in the control chamber.

[3]Mean percentage of mosquitoes which left the release chamber.

*Significant difference ($P < 0.01$) of mean percentage in test- and control chamber: *t*-test for paired samples. Within all experiments means in the test- or active columns followed by the same letter are not significantly different (P 0.05, one-way ANOVA: LSD *post hoc* test).

When ammonia was added to a combination of either one with lactic acid, a significant increase of attractiveness was observed only with fraction I, but not with fraction II (experiment 5, Table 1). The combination of fraction I, lactic acid and ammonia, however, was less attractive than the combination of fractions I and II and lactic acid.

## Discussion

The data presented here clearly demonstrate an attractive effect of ammonia on *Ae. aegypti* in concentration ranges which exist around or downwind from human hosts.

The concentration of this compound in human breath has been found by several investigators to be between 120 and 3170 ppb (Larson *et al.*, 1979; Norwood *et al.*, 1992). The lowest concentration which caused a significant behavioural response in the olfactometer was found to be 0.7 nmol/l air (17 ppb), which is clearly below the concentration of ammonia in breath. Another major source of ammonia is the human skin. Sweat produced by the eccrine sweat glands contains 0.7–25 mmol/l (12–425 mg/l) ammonia and 3.9–67.7 mmol/l (235–4000 mg/l) urea, which is quickly decomposed to ammonia by the bacterial microflora on the skin surface (Fiedler, 1968; Ciba-Geigy, 1977). The high lactic acid concentration (27–37 mmol/l = 2.5–3.4 g/l) of human sweat sets the pH value of human skin between 5 and 6.8 (Fiedler, 1968). At this pH value most of the ammonia is bound as salts and composes a buffer system together with lactate/lactic acid. Since we do not know the evaporation rate of gaseous ammonia above the human skin surface, we tested an aqueous solution of ammonium chloride (pH 6.5) and an aqueous lactic acid/ammonia buffer (pH 5) in con- centration ranges similar to that found in human sweat. Both stimuli significantly enhanced the responses to lactic acid, indicating a considerable evaporation of ammonia from these sources. These results and also the dose–response characteristics (Figure 3) indicate that *Ae. aegypti* is sensi- tive to ammonia at the levels which are given off by humans with their breath as well as from their skin.

From our data, we assume a sensory threshold to am-

monia in a concentration range between 2 and 17 ppb, which is similar to the one found in the haematophagous bug *Triatoma infestans* (Taneja and Guerin, 1997). Nymphs of these bugs were attracted to concentrations of 3 and 17 ppb on a servosphere, whereas no significant response was found at 0.3 ppb. Other examples of attraction or aggregation to ammonia sources have been documented for a variety of both haematophageous and non-haematophagous arthropods, such as the horse-fly *Hybomitra lasiophtalma* (Hribar *et al.*, 1992), the human body louse *Pediculus humanus* (Mumcuoglu *et al.*, 1986), the cockroach *Blatella germanica* (Sakuma and Fukami, 1991) and the Mediterranean fruit fly (Mazor *et al.*, 1987). Female fruit flies use ammonia as an attractive odour cue in a similar context as female *Aedes* mosquitoes: they are attracted towards ammonia-releasing proteinaceous sources in order to retrieve protein for egg maturation. In contrast to yellow fever mosquitoes, which respond to ammonia only in combination with lactic acid, fruit flies and *Triatoma* bugs are attracted by ammonia alone (Taneja and Guerin, 1997). This might reflect the different behavioural contexts in which ammonia is used by mosquitoes with their narrow host range on the one hand and by more opportunistic insects on the other. Only in combination with a specific human skin component such as lactic acid might ammonia contribute to the host recognition of the anthropophilic mosquito *Ae. aegypti*. The opportunistic bug *T. infestans*, however, might make more versatile use of the same stimulus, e.g. for finding their refuges, which are marked with ammonia-releasing faeces, and also for host finding (Taneja and Guerin, 1997). The finding that ammonia is attractive to yellow fever mosquitoes only in combination with lactic acid explains the results of Brown (Brown, 1952), Rössler (Rössler, 1961) and Müller (Müller, 1968), who could not find behavioural responses to ammonia stimuli because they did not test this compound together with lactic acid.

Previous studies on *Ae. aegypti* in our laboratory showed that enzymatic decomposition of lactic acid abolished the attractive effect of human skin residues (Geier *et al.*, 1996). An attractive effect was regained by combining synthetic lactic acid with the lactic acid-deprived residues. This implies that all components which contribute to the attractiveness of skin odour are only effective when lactic acid is present concurrently. The observed behavioural responses to ammonia correspond with these findings. Lactic acid seems to play the key role in odour-mediated host finding of yellow fever mosquitoes. It acts synergistically together with carbon dioxide, as well as with ammonia and other unidentified compounds on human skin. The fact that no synergistic effects were found between ammonia and carbon dioxide shows that neither ammonia nor carbon dioxide can substitute lactic acid as a synergist in attracting *Ae. aegypti*. Therefore we suggest an olfactory host recognition pattern in which different compounds of the attractive odour might act together at distinct levels of synergism. The highly attractive skin extract contained, beside many other compounds, considerable amounts of free lactic acid (6mmol/l) and ammonia (7.4 mmol/l). While the complete skin extract attracted 80–90% of the mosquitoes, a mixture of lactic acid and ammonia attracted at most 45%. Since this attractiveness did not increase even with higher doses of ammonia, it is obvious that additional components of the extract play a role. This is further confirmed by the results from the combinations of two behaviourally active fractions of the skin extract with ammonia. Both fractions and lactic acid combined are as effective as an equivalent amount of skin extract. Ammonia increased the attractiveness of fraction I plus lactic acid whereas no increase was observed with fraction II plus lactic acid. The combination of fraction I, lactic acid and ammonia, however, was less attractive than the combination fractions I and II and lactic acid. This suggests that ammonia is an effective principal in fraction II, but it is obviously not the only one.

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# INFLUENCE OF ODOUR PLUME STRUCTURE ON UPWIND FLIGHT OF MOSQUITOES TOWARDS HOSTS

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## Summary

**Both the concentration and the fine-scale plume structure of host odours influence the upwind flight of female mosquitoes *Aedes aegypti* (L.) (Diptera: Culicidae) in a wind tunnel. The attractive effects of carbon dioxide, human skin odour and L-(+)-lactic acid were tested in homogeneous, turbulent and filamentous odour plumes. With carbon dioxide, the percentage of upwind-flying mosquitoes increased with the increasing fluctuations in concentration that occur in turbulent and filamentous plumes. In homogeneous plumes, an initial activation effect was observed, but sustained upwind flights were less frequent than in the other plumes. The opposite was found with plumes of human skin odour: the highest number of mosquitoes flew upwind in the homogeneous plume, whereas in turbulent or filamentous plumes their numbers were significantly lower. Regardless of plume type, the percentage of upwind-flying mosquitoes increased with increasing concentrations of carbon dioxide and of skin odour. With L-(+)-lactic acid, the dose–response characteristics were not consistent, and the relative effects of different plume types upon upwind flights differed within different ranges of concentration. Even maximum reactions to this compound were modest compared with those to carbon dioxide or to skin odour. Our findings demonstrate (1) that mosquitoes are able to orient upwind under continuous odour stimulation and (2) that upwind flight is dependent upon plume structure in different ways for different host odour components.**

Key words: *Aedes aegypti*, mosquito, host-finding, upwind flight, odour plume, attraction, carbon dioxide, lactic acid.

## Introduction

The orientation of insects towards attractive odour sources depends upon both the temporal and spatial distribution of odorants downwind from the source. This was first investigated by Wright (1958) and studied in further detail using male moths, which are attracted by female pheromones over long distances (Baker and Vickers, 1997; Cardé and Mafra-Neto, 1997; Murlis et al., 1992; Kaissling and Kramer, 1990). An odour evaporating from a small source such as a female pheromone gland remains confined to a thin filament of pheromone-laden air near the source. During downwind transport, however, the filament becomes increasingly disrupted and diluted due to turbulence in the air. This results in a highly intermittent signal, whose temporal and structural characteristics vary with distance from the source (Murlis, 1997; Murlis and Jones, 1981). For the males of several moth species, such intermittence has been demonstrated to be necessary for sustained upwind progress (Kennedy et al., 1980, 1981; Willis and Baker, 1984; Baker et al., 1985; Kramer, 1986, 1992; Kaissling and Kramer, 1990). More recent experiments demonstrate that the brief moments of perception of short pheromone pulses dictate subsequent flight manoeuvres (Mafra-Neto and Cardé, 1994, 1995, 1996; Vickers and Baker, 1994; Baker and Vickers, 1997). Although many aspects of odour-mediated orientation have been studied in other flying insects, such as parasitoid wasps (Kaiser et al., 1994; Kerguelen and Cardé, 1997) and haematophagous Diptera (Brady et al., 1989, 1995; Bursell, 1984, 1990; Griffiths and Brady, 1995), we know little about the influence of the fine-scale structure of odour plumes in these cases. It would be interesting to learn whether different adaptations exist in other cases of odour-guided orientation that occur in different contexts and under different stimulus conditions, such as the host-finding behaviour of bloodsucking insects. There are no quantitative data regarding the spatial/temporal distribution of odours emitted by the potential hosts of such insects. However, we can assume that, in contrast to the typical pheromone plume arising downwind from a small source such as a female moth's pheromone gland, the odours released from a larger source such as a human body will form an initially large and probably less disrupted plume with different fine-scale characteristics (Cardé, 1996). Other characteristic features of the distribution of odours around or downwind of larger animals result because odours are emitted in two different ways (1) with the breath, which is exhaled

periodically, and (2) from the skin, which gives off volatile substances continuously by convection currents.

As a first step in exploring whether the plume structure influences the odour-mediated orientation of mosquitoes, we investigated how differences in the spatial/temporal distribution of odour molecules downwind from an odour source affect the upwind flight of female *A. aegypti* towards different components of host odour in a wind tunnel. We used carbon dioxide, L-(+)-lactic acid and a natural blend of human skin components as test odours. The latter has repeatedly been shown to attract mosquitoes in different types of olfactometers (Rahm, 1958; Schreck et al., 1981; summary in Takken, 1991; Geier et al., 1996). Carbon dioxide, a major component of exhaled breath, is known to increase the probability of take-off and to prolong the duration of flight in mosquitoes; it is also an attractant (Geier et al., 1996; Eiras and Jepson, 1991; Gillies, 1980). L-(+)-Lactic acid can be detected both in human breath and on human skin (Acree et al., 1968; Smith et al., 1970). As a single stimulus, this compound is only slightly attractive for *A. aegypti*, but in combination with carbon dioxide it acts as a synergist by increasing the total attractiveness (Acree et al., 1968; Smith et al., 1970; Eiras and Jepson, 1991). L-(+)-Lactic acid also plays an essential role in the attractiveness of human skin odour since without this compound the remaining volatiles from the skin are not effective (Geier et al., 1996).

Odour plumes of three different structures were generated by varying the mode of odour inlet into the air current of the wind tunnel. The resulting distribution of odours in the plumes was simulated and visualised by introducing smoke from $TiCl_4$ sources instead of odorants into the wind tunnel.

## Materials and methods

### *Animals*

10- to 40-day-old female *Aedes aegypti* (L.) from cultures raised at the Centre for Plant Research (*Pflanzenschutzzentrum*) at Bayer AG, Monheim, Germany, were used in our experiments. They were reared as larvae and fed with Tetramin fish food. Adults (300–500) were kept in containers (50 cm×40 cm×25 cm) at 26–28 °C, 60–70 % relative humidity and on a 12 h:12 h L:D photoperiod. In the containers, the animals had access to a 10 % glucose solution on filter paper.

### *Wind tunnel*

The wind tunnel consisted of an 800 mm long transparent Plexiglas tube (thickness 5 mm). The tube was 70 mm in diameter, representing approximately seven *A. aegypti* wingspans. A gauze screen situated 150 mm from the upwind end of the tunnel could be rotated by hand to open or close the upwind chamber (Fig. 1A). The wind tunnel connected with a polyvinylchloride tube (stimulus chamber, length 200 mm, inner diameter 100 mm) at the upwind end, where the odour plumes were generated. The release chamber (length 150 mm; inner diameter 70 mm) containing the mosquitoes was attached to the downwind end of the wind tunnel. A constant air stream (flow rate 58 l $min^{-1}$) from the Institute's pressurised air system was purified using an activated charcoal filter, heated and humidified before being passed through the wind tunnel. Further details of this experimental arrangement are described elsewhere (Geier, 1995). The temperature in the wind tunnel was 28±1 °C, the relative humidity 70±5 % and the wind speed 0.25 m $s^{-1}$.

The wind tunnel was placed on a white table, and a white cardboard shield (height 20 cm) covered both sides of the wind tunnel. Overhead illumination was provided by two 40 W light bulbs.

### *Plume generation*

Different patterns of odour distribution in the wind tunnel air stream were generated by injection of odorants into the stimulus chamber at different positions (Fig. 1B). Stimulus-laden air was injected through Pasteur pipettes (tip i.d. 1.1 mm, o.d. 1.4 mm, total length 150 mm, length of tip 60 mm) at flow rates of 1600 ml $min^{-1}$ at position 1 and 200 ml $min^{-1}$ at positions 2 and 3 (see Fig. 1B). In this way, three distinctly different patterns of odour distribution were produced, which were simulated and visualised (see Fig. 4A) by introducing smoke into the tunnel instead of odours. To generate smoke, 0.1 ml of $TiCl_4$ (99 %, Merck) was placed on a strip of filter

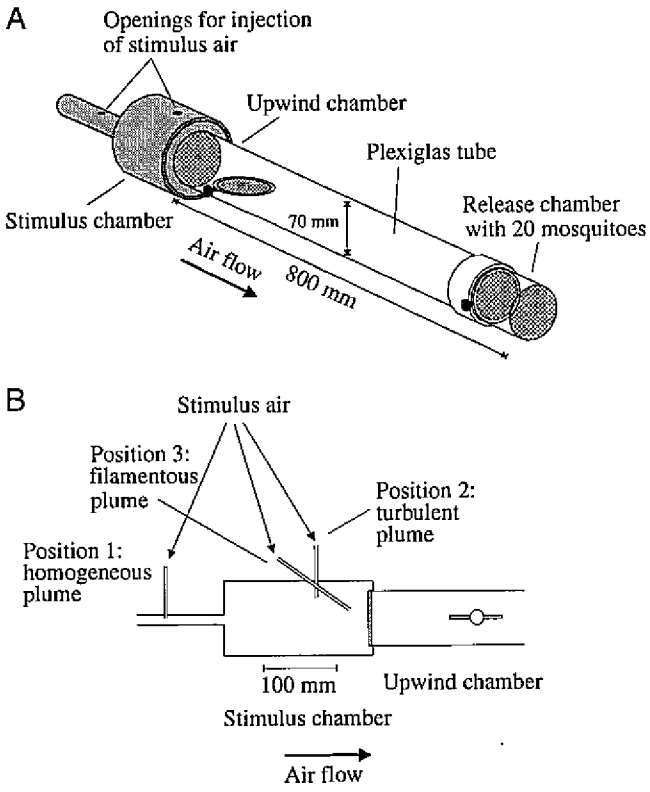


Fig. 1. (A) Diagram of the wind tunnel. (B) Section through the stimulus chamber. Different plume structures were produced by injecting stimulus air *via* Pasteur pipettes at three different positions.

paper (0.5 cm×5 cm), which was then introduced into the pipette. When air was passed through the pipette, plumes of smoke, whose structures varied considerably according to the different positions of the pipettes and the different flow rates, appeared in the wind tunnel (Fig. 1B, see Fig. 4A). To estimate the temporal distribution of odours within the different plume types, the smoke density was measured using small light barriers placed in the middle of the wind tunnel at a distance of 10 cm and 70 cm, respectively, from the upwind end. These light barriers consisted of an infrared light-emitting diode facing an infrared phototransistor (distance 15 mm). The diameter of the infrared beam was 1.5 mm. Smoke blown across the beam changed the voltage produced by the phototransistor, and this was taken as a measure of smoke density. The voltage signals were amplified, fed into an analogue-to-digital (A/D) converter (Syntech IDAC-01) at rate of 6944.4 samples $s^{-1}$, stored on a personal computer and analysed using Autospike software (Syntech, Hilversum, The Netherlands). The number of smoke peaks per unit time and their amplitude were evaluated. Peaks were counted where the amplitude was 50 % higher than the background level and where they occurred more than 25 ms apart.

### *Odour stimuli*

Three different odour stimuli were used; in addition, compressed air purified with a charcoal filter was used as control stimulus to check for contamination effects. L-(+)-Lactic acid at varying concentrations was produced with an olfactometer based on the design of Ough and Stone (1961). Charcoal-filtered compressed air at flow rates from 0.16 ml $min^{-1}$ to 1000 ml $min^{-1}$ was passed through a 250 ml Erlenmeyer flask filled with 10 ml of L-(+)-lactic acid solution (90 % aqueous solution; Merck). The air stream passed over the surface of the solution. To determine the output of lactic acid, the air stream was passed at various flow rates through solutions of 0.01 mol $l^{-1}$ sodium hydroxide and titrated with 0.01 mol $l^{-1}$ HCl solution (Ough and Stone, 1961). Fig. 2 shows the relationship between the flow rate through the flask and the amount of lactic acid trapped per minute. To ensure that the total output of lactic acid was collected, four sodium hydroxide traps were used in series, and the amount in each trap was determined. We never found significant amounts of lactic acid in the last trap. Carbon dioxide was taken from a gas cylinder with a trade-standard purity of 99.9 % (Linde, Germany). Various volumes of the gas were mixed with clean air to produce different concentrations in the stimulus air. According to the method of Schreck et al. (1981, 1990), skin odours from a volunteer subject were transferred to the surface of a glass test tube (o.d. 16 mm, length 160 mm) by intensely rubbing it in the hands for 5 min. The hands were rinsed with tap water 2 h before the experiment, and care was taken to avoid any contact with cosmetics or perfumes prior to the rubbing. The glass tube with attached skin residues was then inserted into a Teflon tube (i.d. 17 mm, length 150 mm), and charcoal-filtered air was passed through the narrow space between the Teflon tube and the glass tube at 1600 ml $min^{-1}$

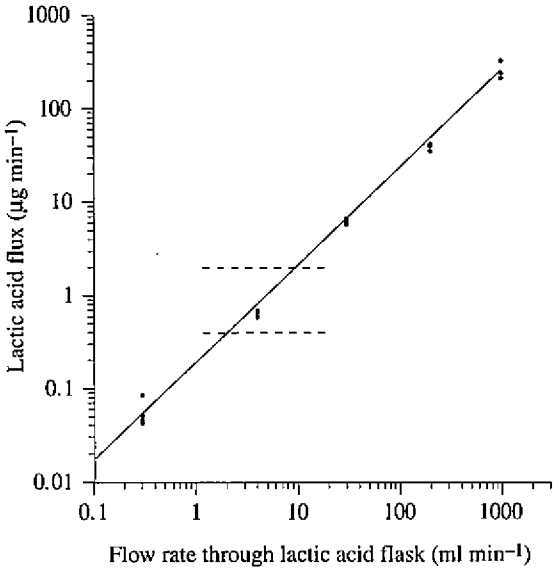


Fig. 2. Calibration of the lactic acid output from the stimulus flask. Each point shows the amount of lactic acid trapped per minute in a solution of 0.01 mol $l^{-1}$ sodium hydroxide at a given flow rate. Flow rates between 0.3 and 1000 ml $min^{-1}$ were used. The regression line was used to calculate the lactic acid flux at flow rates lower than 0.3 ml $min^{-1}$ by extrapolation. The equation for the regression line was $\log y = -0.757 + 1.041 \log x$ ($r^2$=0.996, $P$<0.0001). The broken horizontal lines indicate the lowest and the highest rates of lactic acid output from human hands measured by Smith et al. (1970).

(Fig. 3). To vary the odour concentration in the plumes, the odour-laden air was mixed with clean air. The concentration of the skin odour in the plume was defined as the ratio of air loaded with skin odour to the volume of the total air stream forming the plume.

Flow meters (for flow rates over 3 ml $min^{-1}$) or a precision tubing pump (Masterflex, Novodirect GmBH, Kehl/Rhein, Germany; flow rates 0.1–3 ml $min^{-1}$) were used to control the gas flow.

To estimate the odour concentration in the plumes, the maximum possible concentration of the generated odour filaments was used as a reference value. For the turbulent and filamentous plumes, this concentration was considered to be the same as in the injected stimulus-laden air. For homogeneous plumes, in which the injected odours are evenly distributed, the concentration was calculated from the injected dose and the total volume of air in the wind tunnel.

### *Bioassay*

Groups of 18–22 female mosquitoes were used for the tests. The experimenter's hand was used to lure them out of their cage into the release chamber, which then was attached to the downwind end of the wind tunnel. Before odour stimulation, the mosquitoes were given 20 min of acclimation time in the wind tunnel air stream. Upon stimulus onset, the release chamber was opened and the mosquitoes were allowed to enter the wind tunnel. After 30 s of stimulation, the rotating screens

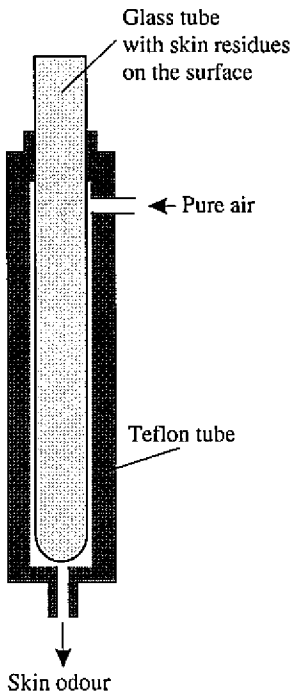


Fig. 3. Apparatus for generating skin odour stimuli. A glass test tube, which had been rubbed intensively in the hands of a volunteer subject, was placed into a Teflon tube. An airflow of 1600 ml $min^{-1}$ was passed through the gap (0.5 mm) between the glass tube and the wall of the Teflon tube. The exiting air contains skin odour, which can be diluted with clean air to provide various stimulus concentrations.

of both the upwind and the release chamber were closed, and the number of mosquitoes that had left the release chamber and the number of mosquitoes that had reached the upwind end were counted. After the experiment, the wind tunnel was connected to a fan which reversed the airflow (wind speed approximately 0.3 m $s^{-1}$). The mosquitoes were then lured back into the release chamber using the hand as an attractant. The wind tunnel was then reattached to the purified air stream. Each group of mosquitoes was tested up to 10 times with an interval of at least 20 min between each test.

Each odour was tested in a separate block of experiments. Within such a block, both stimulus concentration and plume type were varied. Each stimulus treatment and pure air as a control were tested using 20 different groups of mosquitoes, each group being exposed to every treatment in random order. Between the tests, a constant flow of fresh air was passed through the wind tunnel. The experiments were carried out between 09:00 h and 18:00 h.

### *Evaluation*

In each test, we distinguished two behavioural categories of responses: (1) the percentage of mosquitoes found outside the release chamber was taken as a measure of activation, which included flight initiation and short-duration upwind progress; (2) the percentage of mosquitoes trapped at the upwind end after 30 s was taken as a measure of sustained upwind flight. The values for both activation and upwind flight were averaged from the 20 experiments. Since the data are percentages, they were transformed using angle transformation (Sokal and Rohlf, 1981) for further statistical analysis. The transformed means were analysed independently using a one-way analysis of variance (ANOVA) with the least-squares difference (LSD) method as a *post-hoc* test for comparison of treatments. All calculations and statistics were carried out using SPSS 6.0 for Windows.

## Results

### *Features of odour plumes*

The different types of odour plumes generated in the wind tunnel were visualised using plumes of $TiCl_4$ smoke (Fig. 4A). The distribution of smoke was verified several times with consistent results during the course of the study. The smoke pattern shows that the plume structure is mainly dependent upon (1) the position of the injection pipette and (2) the injected volume of air. We found empirically that injection of 1600 ml $min^{-1}$ at position 1 (Fig. 1B) resulted in the formation of a homogeneous plume (Fig. 4A), uniformly distributed over the whole cross section of the wind tunnel. No gradient appeared along the wind tunnel, as documented by the photographs and by the measurements of smoke density (Fig. 4B). This indicates that odours applied in this way were evenly distributed in the wind tunnel. Injection of smoke at 200 ml $min^{-1}$ at position 2 (Figs 1B, 4) generated a turbulent plume, which is unevenly distributed, with filaments or areas of higher smoke density alternating with areas of lower density. These variations are smaller than those found in the filamentous plume, with the plume relatively dispersed in the wind tunnel. Measurements of smoke density at both ends of the wind tunnel revealed that the plume became less disrupted and more evenly distributed towards the downwind end. A filamentous plume type was produced by the injection of 200 ml $min^{-1}$ at position 3 (Fig. 1B). The oblique position of the glass pipette resulted in the formation of a meandering filament, which breaks down into filaments and packets (Fig. 4). Such filaments of high smoke density alternate with pure air, and the distribution of smoke is highly intermittent. As in the turbulent plume, the filaments were more dispersed and diluted towards the downwind end. To quantify the difference in the plume structures, the smoke density was measured for 20 s in three experiments using the infrared light-emitting diodes. In the homogeneous plume, no peaks of high smoke density were detected. The turbulent plume yielded peaks with a mean amplitude of 4.7±1.0 mV ($N$=96). The mean frequency of pulses was 1.6±1.1 Hz ($N$=3) and the mean duration of pulses was 55.3±26.8 ms ($N$=96). The mean peak amplitude in the filamentous plume was 35.4±35.4 mV ($N$=339), the frequency 5.7±2.3 Hz and the mean duration of pulses 83.8±50.6 ms ($N$=339) (means ± S.D.). The turbulent and filamentous plumes differed significantly in pulse duration

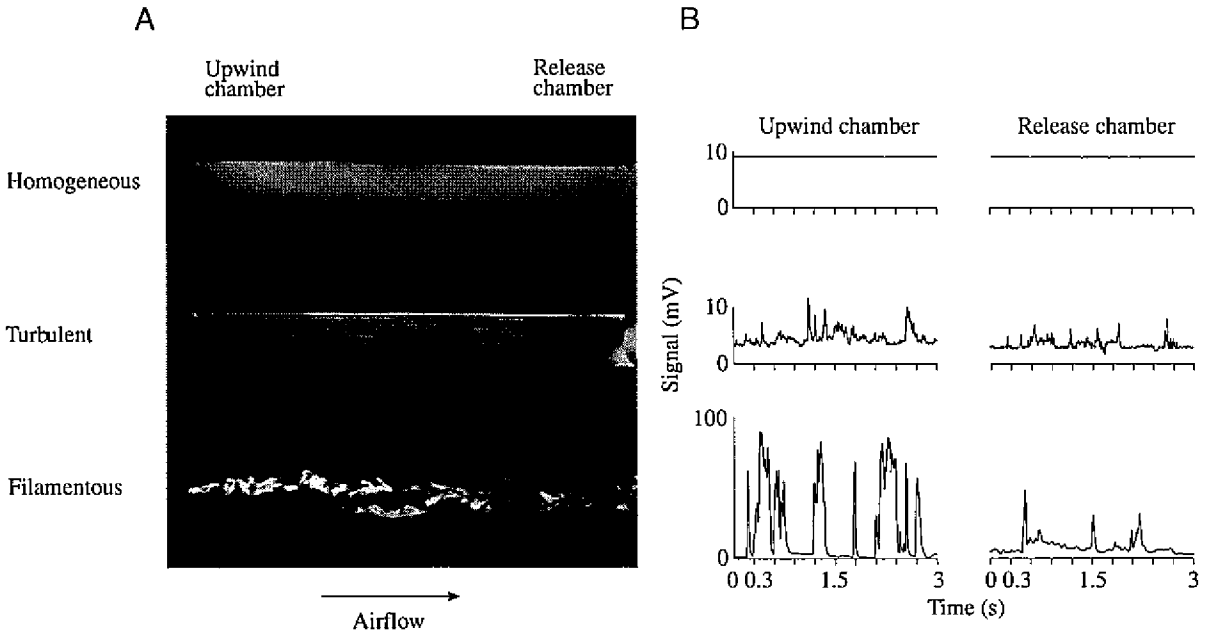


Fig. 4. Smoke visualisation of odour plumes in the wind tunnel. (A) Photographs of the wind tunnel from above during the generation of homogeneous, turbulent and filamentous plumes (see Fig. 1B) using $TiCl_4$ smoke in place of an odour stimulus. The photographs were taken 10–15 s after 'stimulus' onset. The wind tunnel was illuminated from the left side. (B) Local smoke densities measured in each plume type using two small light-emitting diodes and transistors placed in the centre of the wind tunnel, one 10 cm from the upwind end (upwind chamber) and one 70 cm from the upwind end (release chamber). Changes in smoke density caused changes in the voltage output of the phototransistor. Note that the voltage scale differs for the filamentous plume.

($t$=3.06; $P$=0.003; $t$-test for unpaired samples) and pulse amplitude ($t$=7.65; $P$<0.0001; $t$-test for unpaired samples).

### *Behaviour of the mosquitoes in the wind tunnel without odour stimuli*

During the first minutes after attaching the release chamber to the wind tunnel, most of the mosquitoes flew around in the chamber. They gradually became more stationary, and after 20 min of acclimation they were positioned on the gauze or on the wall of the release chamber, with flights occurring only sporadically. After opening the rotating screen, 20–40 % of the mosquitoes left the chamber during the 30 s test period in control tests with pure air (Fig. 5). These mosquitoes mostly flew a short distance upwind, turned back, flew upwind again, and so on. Sometimes they landed on the wall of the wind tunnel, and in a few cases they flew back into the release chamber. The mean percentage of mosquitoes trapped after 30 s in the upwind chamber was 10 % or less (Fig. 5). The activation response did not differ significantly between the different plume types, but the percentage of mosquitoes that reached the upwind end was slightly, but significantly, greater for the filamentous plume than for the homogeneous or turbulent plume.

After all the tests, the mosquitoes could easily be lured back

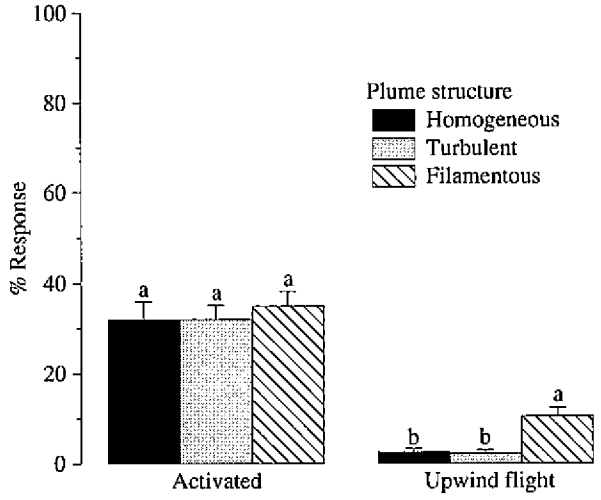


Fig. 5. Responses of mosquitoes to pure air. Three plume types (homogeneous, turbulent and filamentous) of pure air were tested by injecting air without addition of odours into the stimulus chamber (see also Fig. 1B). The shading of the columns indicates the plume type. 'Activated' represents the mean percentage of mosquitoes that left the release chamber. 'Upwind flight' represents the mean percentage of mosquitoes trapped in the upwind chamber of the wind tunnel after 30 s. Values are means + S.E.M. of 20 experiments. In each experiment, 18–22 mosquitoes were tested. Means within each category were compared using an LSD *post-hoc* test and a one-way ANOVA; different letters above columns indicate significant ($P$<0.05) differences within a category.

into the release chamber by using the experimenter's hand as an attractant and reversing the airflow in the wind tunnel. The initially increased flight activity that occurred immediately after exposure to the hand odour decreased during the following rest period of 20 min, during which the mosquitoes were exposed to clean wind tunnel air only. Using such 'recovery' intervals, we found no indication that repeated stimulus exposure affected the responses of mosquitoes in subsequent tests.

*Response to carbon dioxide*

To investigate the effects of carbon dioxide, 20 groups of mosquitoes were tested with homogeneous, turbulent and filamentous plumes of carbon dioxide varying in concentration from 0.05 to 100 vol%. The maximum concentration tested in the homogeneous plume structure was 4 % to avoid toxic effects. At all concentrations, a significantly higher percentage of mosquitoes reached the upwind chamber in the filamentous plume than in the other plume types, and the turbulent plume also evoked a significantly higher upwind-flight response than the homogeneous plume at a concentration of 4 % (Fig. 6A). Two trends are obvious for sustained upwind flight: (1) in all plume types, the response increased with increasing concentration of carbon dioxide; (2) the response also increased with increasing fluctuation of carbon dioxide concentration within the wind tunnel air. These trends are not consistent for the activation response, with the response to the homogeneous plume being significantly greater at low levels of carbon dioxide (Fig. 6A). The homogeneous carbon dioxide plume appeared to stimulate the mosquitoes to take flights with a short-duration subsequent upwind progress. However, they did not fly persistently upwind, often changed their flight direction and flew around irregularly. Some mosquitoes flew into the upwind chamber within the first few seconds, but then flew out again during the experiment. This was rarely observed with turbulent or filamentous plumes. In general, the carbon dioxide stimuli increased the percentage of activated and of upwind-flying mosquitoes compared with the control experiments with pure air (Figs 5, 6A).

*Response to lactic acid*

Compared with the control experiments, the lactic acid odour slightly increased the percentage of upwind-flying mosquitoes, but an influence of plume structure on activation and upwind flight was less obvious (Fig. 6B). At lower doses, the homogeneous plume stimulated a similar percentage of upwind-flying mosquitoes to the turbulent or filamentous plumes at higher doses. Whereas approximately 60 % of the mosquitoes flew upwind in the filamentous carbon dioxide plume at the highest concentrations (Fig. 6A), no more than 20 % flew upwind in lactic acid plumes regardless of the plume structure. Homogeneous plumes were more effective than turbulent or intermittent ones for lower concentrations of lactic acid. The responses to the more fluctuating plumes increased with increasing concentration of lactic acid; however, a significant decline in the number of upwind-flying mosquitoes occurred at the highest lactic acid concentration in homogeneous plumes. This decline was not seen in the turbulent or filamentous plumes, even though the maximum concentration used was approximately six times higher than in the homogeneous plume. In general, lactic acid at the tested concentrations activated the mosquitoes to a lesser degree than did carbon dioxide, and the plume structure did not consistently affect this behavioural response.

*Response to odours from skin residues*

The upwind-flight response to skin odour depends significantly both on the odour concentration and on the fine-scale structure of the plume (Fig. 6C). The percentages of activated and of upwind-flying mosquitoes were significantly higher for the homogeneous skin odour plume than for the turbulent and filamentous plumes. This effect was also apparent in the different slopes of the dose–response relationships between the homogeneous plume and the turbulent or filamentous plume. For a 100-fold increase in odour concentration, the behavioural responses increased from 40 % to 95 % (activation) and from 10 % to 87 % (upwind flight) in the homogeneous plume. For a 200-fold increase in concentration, however, filamentous and turbulent plumes evoked only an increase of the behavioural responses from 40 % to 60 % (activation) and from 10 % to 30 % (upwind flight). The responses in turbulent and filamentous plumes did not differ significantly.

## Discussion

*Plume features*

Our findings regarding the influence of the distribution pattern of host odours upon the upwind flight of *A. aegypti* rely upon the applicability of the patterns visualised using $TiCl_4$ smoke instead of odour. The use of smoke to visualise odour patterns is generally accepted, since it is assumed that the plume's physical dimensions and structure are essentially independent of the chemical and physical properties of the material within it (Murlis et al., 1992). In wind-tunnel experiments with male gypsy moths *Lymantria dispar*, however, Charlton et al. (1993) obtained different estimates of the size of a pheromone plume at high concentrations from behavioural responses (wing-fanning) and smoke visualisation. Therefore, we should not overestimate the precision of the patterns derived from smoke visualisation when correlating plume features with behavioural responses. Nevertheless, smoke visualisation provides at least a qualitative measure of the distribution of odours and permits differentiation between homogeneous, turbulent and filamentous plumes.

Since the odour filaments in turbulent or filamentous plumes were not precisely defined and were dispersed irregularly, the odour intensity perceived by a mosquito flying through the wind tunnel might vary considerably and cannot be determined exactly. The resolution of the smoke densities measured with the light-emitting diode does not permit an estimation of the odour density in the filaments, because the density is averaged

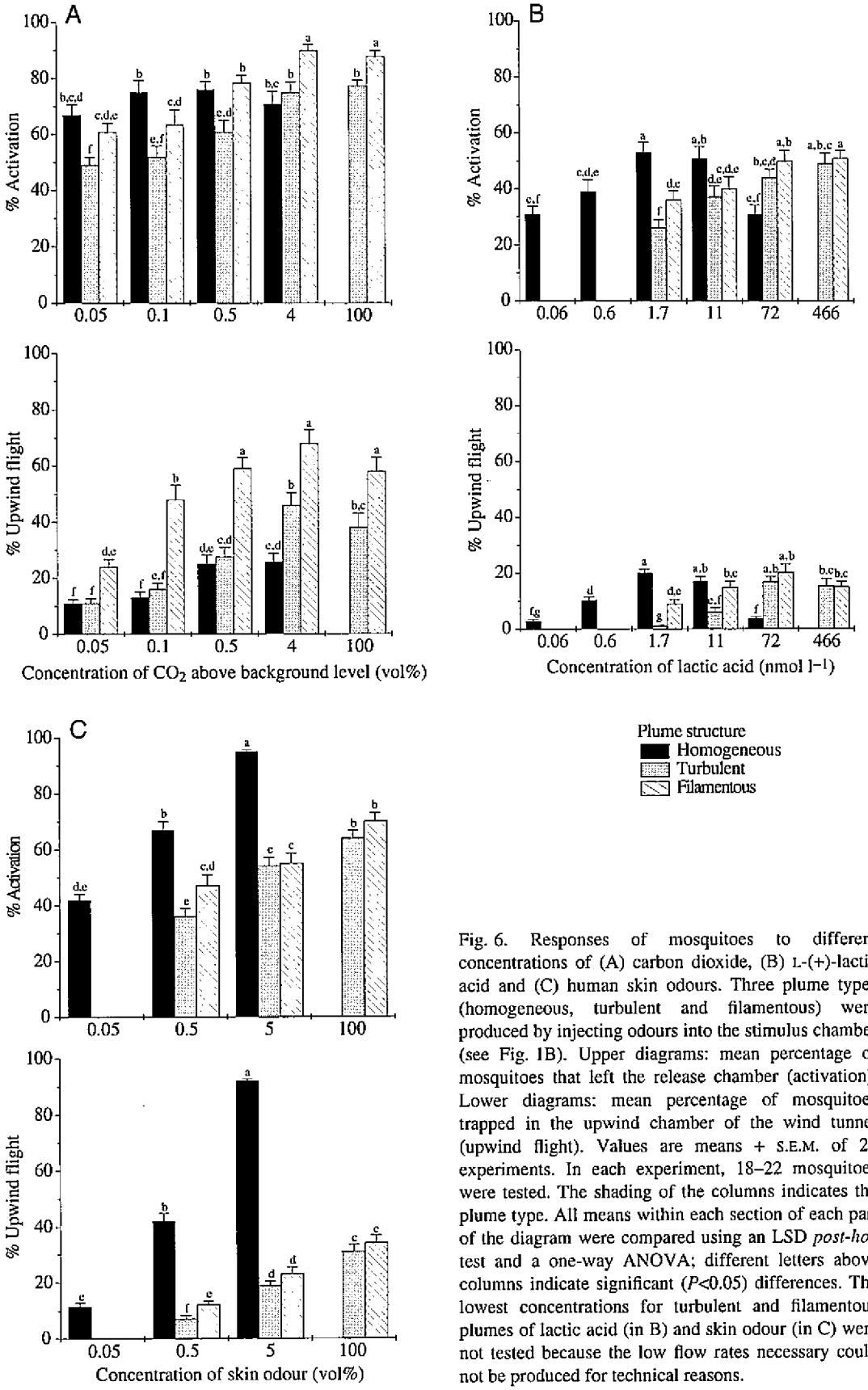


Fig. 6. Responses of mosquitoes to different concentrations of (A) carbon dioxide, (B) L-(+)-lactic acid and (C) human skin odours. Three plume types (homogeneous, turbulent and filamentous) were produced by injecting odours into the stimulus chamber (see Fig. 1B). Upper diagrams: mean percentage of mosquitoes that left the release chamber (activation). Lower diagrams: mean percentage of mosquitoes trapped in the upwind chamber of the wind tunnel (upwind flight). Values are means + S.E.M. of 20 experiments. In each experiment, 18–22 mosquitoes were tested. The shading of the columns indicates the plume type. All means within each section of each part of the diagram were compared using an LSD *post-hoc* test and a one-way ANOVA; different letters above columns indicate significant ($P<0.05$) differences. The lowest concentrations for turbulent and filamentous plumes of lactic acid (in B) and skin odour (in C) were not tested because the low flow rates necessary could not be produced for technical reasons.

over a volume of 0.4 $cm^3$. Furthermore, differentiation between a thin smoke filament with high density and a thicker one with lower density is not possible. However, the smoke distribution shown in Fig. 4A and the peak values of the smoke density measurements plotted in Fig. 4B suggest that odour clusters of higher concentration exist more often in filamentous than in turbulent plumes. In homogeneous plumes, odours are equally distributed, and the actual odour concentration is the same throughout the wind tunnel. However, because a flying insect is not stationary relative to the air, the flux of odour molecules measured by the sensory receptors will vary depending on the direction and speed of flight with respect to the air currents even at a constant wind velocity and stimulus concentration (Kaissling and Kramer, 1990; Elkinton and Cardé, 1984). To compare concentration effects within the three plume types, the maximum concentration that mosquitoes could encounter within a plume was taken. In the homogeneous plume, this value is constant throughout the wind tunnel, whereas in filamentous and turbulent plumes, the peak concentration decreases during downwind transport. Since it is difficult to define the exact stimulus strength detected by the mosquito during an experiment, this variable was changed for all three plume types by changing the concentration of the injected stimulus air. This allowed discrimination between the effects of odour concentration and the influence of short-term fluctuations in the signal within the odour plume.

*Effects of different plume structures*

The tests using plumes of carbon dioxide demonstrate that increased intermittence of the signal increased the probability of sustained upwind flight, irrespective of concentration over a wide range. The large difference between the percentage of activated mosquitoes and the percentage of mosquitoes found in the upwind chamber in homogeneous plumes indicates that upwind progress is reduced under continuous stimulation with carbon dioxide. This is further confirmed by our observations of irregular up- and downwind flights in the homogeneous plume. These findings are in agreement with the proposed orientation mechanisms of male moths following upwind a plume of sex pheromone released from a calling female. The intermittence of the pheromone signal is essential for the sustained upwind flight of male moths (Kennedy et al., 1980, 1981; Willis and Baker, 1984; Baker et al., 1985; Kaissling and Kramer, 1990). Recent studies have shown that the fine-scale structure of a pheromone plume influences instantaneous flight manoeuvres (Mafra-Neto and Cardé, 1994, 1995, 1996; Vickers and Baker, 1994; Baker and Vickers, 1997). Omer (1979) has demonstrated that carbon dioxide elicits upwind flight of *Anopheles arabiensis* and *Culex pipiens fatigans* only if presented intermittently. In contrast to our experiments, he used a low repetition rate and long-lasting pulses (20 s on, 20 s off). Bowen (1991) argued that it is not the absolute level but the change in concentration of the gas that is the important factor for eliciting behavioural responses. This was confirmed in the present experiments.

Our most surprising result was for stimulation with skin odours. With this stimulus, the percentage of mosquitoes flying persistently upwind was significantly higher in a homogeneous plume than in an intermittent one. This was consistent over a wide range of concentration and was different from the responses to carbon dioxide. These findings provide the first evidence for an alternative orientation mechanism to that proposed in male moths, which need intermittent signals for sustained upwind flight towards a pheromone source. In moths, this is likely to be an adaptation to the special plume pattern that occurs downwind from a calling female (Cardé, 1996). The different responses of mosquitoes to the plumes of carbon dioxide and skin odours might reflect different release patterns of these stimuli from the host. Humans give off carbon dioxide by periodically expiring air through the mouth or nose. It can be assumed that, in the immediate vicinity of such a host, this periodicity and also the turbulence of expired air, which contains 4–5 % carbon dioxide, cause strong concentration fluctuations against the atmospheric background level of 0.03–0.05 %. Accordingly, we found the strongest upwind flight responses in plumes with filaments of concentrations between 0.5 % and 4 %. The odours from the skin, however, are probably given off more continuously and are dispersed by the convection currents produced by a warm-blooded animal (Willemse and Takken, 1994). Moreover, the source size of the odour will have a considerable effect on plume structure: the larger the source, the lower will be the intermittence and the concentration fluctuations (Murlis et al., 1992). Therefore, the distribution of skin odours may be rather homogeneous close to the host and may become more dispersed and intermittent as a result of air turbulence with increasing distance. The observed differences in upwind flight tendency between plumes of carbon dioxide and skin odour may reflect this situation, and two different modes of orientation could be proposed. (1) Far from the host, the mosquitoes perceive a turbulent and intermittent plume, in which both carbon dioxide and skin odours are present. In this disrupted and incompletely mixed plume, synergistic effects of skin odours and carbon dioxide may be important for orientation. The underlying mechanisms could be similar to those found in moths. (2) Near the host, a uniform cloud of skin odours indicates to the mosquitoes that they are near a host and they can then follow the homogeneous plume produced by the skin odour and by the convection currents of a warm-blooded host. It can be assumed that different orientation mechanisms underlie this short-range attraction, and these need to be studied in more detail.

Lactic acid is a major component of both human skin odour and human breath and acts as a synergist together with carbon dioxide and odour components on human skin (Acree et al., 1968; Smith et al., 1970; Geier et al., 1996). According to Smith et al. (1970), the rate of emission of L-(+)-lactic acid from human hands ranges from 0.38 to 2.22 $\mu g\,min^{-1}$. These doses correspond to lactic acid concentrations of 0.08–0.36 $nmol\,l^{-1}$ in the homogeneous plume. We found that the most effective lactic acid concentration was 1.7 $nmol\,l^{-1}$, giving 20 % upwind-flying mosquitoes in the homogeneous

plume. Similar responses were also elicited in turbulent or intermittent plumes, but with filaments of higher stimulus intensity. Homogeneous plumes of these high concentrations of lactic acid resulted in a significant decrease in activation as well as in upwind flights. To understand the behavioural relevance of lactic acid in different plume structures, quantitative investigations of the release patterns in the breath and from the skin should accompany future studies to explore the effects of lactic acid, carbon dioxide and other skin odour components individually and in combination. The responses of *A. aegypti* to different plume structures suggest that the host-finding behaviour of mosquitoes is affected not only by the odour composition but also by the characteristics of the plume structure, which may depend on the shape and body size of the host, on physical variables such as temperature or humidity and on the distribution of distinct components on the body surface. Unfortunately, no quantitative data exist regarding the typical features of odour plumes generated from large vertebrates by breathing or convection currents. The present study was instigated as a first step in the exploration of these aspects of the odour-mediated orientation behaviour of mosquitoes, and we have demonstrated that fine-scale plume structure has a strong influence on the upwind flight behaviour of these insects. Subsequent quantitative behavioural analysis of individual mosquitoes flying in defined odour plumes will be conducted to study their orientation behaviour in detail. In addition to further investigations of the orientation mechanisms, studies must be undertaken of the typical features of plumes generated from the respective hosts of the mosquito species investigated. These findings could also be important for developing traps for mosquito control. For this purpose, different types of odour-release equipment should be tested, which imitate the natural plume pattern given off by a host and thereby enhance the trapping effect of the artificial baits used.

### *Sensory aspects of the perception of intermittent and continuous stimuli*

The peripheral sensory equipment of *A. aegypti* for odour perception has been studied repeatedly (Lacher, 1967; Pappenberger et al., 1996; Davis and Sokolove, 1976; Davis and Bowen, 1994), but we know little about receptor responses with respect to temporal changes of odour stimuli. In addition, the 'follower' capabilities of the carbon dioxide receptors on the maxillary palps to pulsed stimuli have not been studied in detail. Their strong phasic on-response, however, should enable them to follow rapid changes in carbon dioxide concentration (Kellogg, 1970; Grant et al., 1995), as do moth pheromone-specific cells, which can follow up to 10 odour pulses per second (Kaissling, 1986, 1997; Rumbo and Kaissling, 1989). These insects seem to sustain upwind progress only if there is phasic input from the receptor cells. Continuous pheromone stimulation fails to evoke persistent upwind flight probably because of the tonic response of the pheromone chemoreceptor cells or their adaptation (Baker et al., 1988; Kennedy et al., 1980; Willis and Baker, 1984; Kaissling and Kramer, 1990). This might also hold true for the upwind flight of mosquitoes in response to carbon dioxide; for skin odours, the situation is probably different. In response to such odours, *A. aegypti* orient upwind in homogeneous plumes, and it can be assumed (1) that the relevant receptor cells do not adapt quickly to continuous stimulation, and (2) that the tonic responses of these cells are sufficient to evoke sustained upwind flight.

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